"Killer-Cell Immunoglobulin-like Receptor Haplotype Diversity in three Free State population groups"

By
Marius Louw

Masters in Medical Science/Immunology (M.Med.Sc)

Department of Haematology & Cell Biology
Faculty of Health Science
University of the Free State, South Africa

November 2006

Supervisor: Dr André De Kock
Co-supervisors: Prof Vernon Louw, Dr Marius Coetzee

Aan JaHWeH alle eer tot in alle ewigheid!

Acknowledgements

Many thanks to the following people for all their encouragement, friendship and wisdom throughout the duration of this dissertation.

- To my family for their loving support and loyalty.
- Marelie, Liezel, Muriel, and Seb for their friendship, encouragement, and kindness.
- To Emily, Giggles, Elmarie, Marianne and Jan, for their friendship.
- To the staff at the University of the Free State, and the Dept of Haematology and Cell Biology.
- To Dr. André de Kock for his wisdom, guidance, patience and support. Once again many thanks.
- Many thanks to Magda Theron and Christa Coetsee for their encouragement and helpful recommendations throughout the study period.
- My co-supervisors, Prof. Vernon Louw and Dr. Marius Coetzee, many thanks for the support and encouragement.
- Prof. Phillip Badenhorst for allowing me the opportunity to pursue my study.

Without their support this project would not have materialized.

Finally to my mother, who has been the rock foundation in my life, and always there to support me, this project is dedicated to you.

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List of abbreviations

AH	a-haplotypes
AIDS	acquired immunodeficiency disease syndrome
β_2-M	β_2-microglobulin
BH	b-haplotypes
BMT	bone marrow transplants
CAD	caspase-activated deoxyribonuclease
CD	cluster of differentiation
$CD56^{bright}$	cluster of differentiation 56 bright
$CD56^{dim}$	cluster of differentiation 56 dim
χ^2	Chi^2 square
CpG	concentrated phosphodiester-linked cytosine and guanine pairs
CTL	cytotoxic T-lymphocytes
CPP-32	cysteine protease protein 32
ºC	digrees celcius
D	domain
DAP 10	DNA activation protein of 10 kD
DAP 12	DNA activation protein of 12 kD
df	dilution factor
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
FADD	fas-associated death domain
FasR	fas receptor
FasL	fas ligand
Grb2	growth factor receptor bound 2
GvHD	graft vs host disease
GvL	graft vs leukemia
H	histone
HGNC	HUGO Genome Nomenclature Committee
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HLA-A	human leukocyte antigen-A

HLA-B	human leukocyte antigen-B
HLA-C	human leukocyte antigen-C
HLA-DR	human leukocyte antigen-DR
HLA-DP	human leukocyte antigen-DP
HLA-DQ	human leukocyte antigen-DQ
HLA-DR$\alpha\beta_1$	human leukocyte antigen-DR$\alpha\beta_1$
HLA-DR$\alpha\beta_2$	human leukocyte antigen-DR$\alpha\beta_2$
I	iso-leucine
IC	internal control
I-CAD	inhibitory caspase-activated deoxyribonuclease
IDT	integrated DNA technology
IFN-γ	interferon- γ
ig-sf	immunoglobulin-superfamily
IL	interleukin
ILT	immunoglobulin like transcript
ITAMs	immunoreceptor tyrosine-based activation motifs
ITIMs	immunoreceptor tyrosine-based inhibitory motifs
kb	kilo base
kD	kilo dalton
KG	KIR genotype
KIR	killer-cell immunoglobulin-like receptors
KLF	KIR locus frequency
L	leucine
LAIR	Leukocyte-associated inhibitory receptor
LD	linkage disequilibrium
LILR	leukocyte immunoglobulin-like receptor family
LIR	leukocyte inhibitory receptor
LRC	leukocyte receptor complex
µl	micro litre
µM	micro mole
mMole	milli mole
Mb	mega base
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex

MIC	macrophage inhibitory cytokine
min	minutes
mRNA	messenger ribonucleic acid
N-CAM	neural-cell adhesion molecules
NCR	natural cytotoxic receptors
NK	natural killer
NKC	natural killer complex
OD	optical density
r	relative linkage disequilibrium
RCLS	red cell lysis solution
RNA	ribonucleic acid
s	seconds
SC	stromal cells
SH	serum receptor complex homology
SH2	serum receptor complex homology 2
SHP	serum receptor complex homology-containing tyrosine phosphatase
SHIP	serum receptor complex homology-containing inositol 5-phosphatase
Src	serum receptor complex
SSOP-PCR	sequence specific oligo-nucleotide probes-PCR
SSP-PCR	sequence specific primer- PCR
Syk	spleen tyrosine kinase
P	probability
P	pseudo-gene
PCR	polymerase chain reaction
PLC-γ	phospholipase C-γ
pmol	pico mole
Taq	*thermus aquaticus*
TBE	tris-borate-EDTA buffer
TCR	T-cell receptors
TE	tris-EDTA buffer
TM	transmembrane
TNF	tumour necrosis factor

TNF-α	tumour necrosis factor-α
TNFR-I	tumour necrosis factor receptor type-I
TRADD	tumour necrosis factor receptor type-I-associated with death domain
TRAIL	tumour necrosis factor-related apoptosis-induced ligand
UV	ultraviolet
V	valine
Y	tyrosine
ZAP70	zeta chain-associated protein 70 kilo dolton

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CHAPTER 1

Introduction

Killer-cell immunoglobulin-like receptors (KIR) are present on both natural killer (NK) cells as well as on a subset of T-lymphocytes forming part of the natural cytotoxic receptors (NCR) (Harel-Bellan *et al.* 1986).

KIR interacts with human leucocyte antigen (HLA) expressed on the surface of all nucleated cells. Tissue cells which express normal HLA, are resistant to the cytotoxic effects of NK cells. Failure of KIR/HLA interaction renders the abnormal cell susceptible to cytotoxic effects as a result of positive intracellular signals. The resulting intracellular signals affect NK cell activity causing the release of cytotoxic enzymes onto the target cell, which then stimulates programmed cell death in the abnormal cell (Bancroft *et al.* 1993).

KIR is both polymorphic and highly homologous and the genes are located on the long arm of chromosome 19, as part of the leucocyte receptor complex (LRC). KIR genes are tandemly arrayed over a 150 kilo base (kb) stretch of deoxyribonucleic acid (DNA) and normally consist of nine genes (Uhrberg *et al.* 1997). The receptors are named according to the number of extracellular immunoglobulin-like domains (2D or 3D) and the characteristics of the cytoplasmic tail (L or S) (Marsh *et al.* 2003). Long cytoplasmic tails transduce inhibitory signals and possess one or more, immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Short cytoplasmic tails contain charged amino acid residues in their transmembrane region associating KIR with molecules containing immunoreceptor tyrosine-based activation motifs (ITAMs), such as DNA activation protein of 10 kilo doltin (kD) (DAP 10) and DNA activation protein of 12 kD (DAP 12), ultimately transducing an activation signal (Olcese *et al.* 1997).

Although haplotype variation is possible, genes KIR3DL3, KIR2DL4 and KIR3DL2 have been assigned “framework genes” as well as pseudo-gene (P) KIR3DP1 and are present in all haplotypes (Hsu *et al.* 2002). Unique inherited combinations at the alternate KIR sequences constitute characteristic haplotype diversity, which varies

between ethnic cohorts and individuals within a population (Hsu *et al.* 2002). The number of activatory and inhibitory receptors present in the genotype determines the diversity in phenotypic expression, and the relative degree of difficulty with which cytotoxicity will be accomplished.

In this project the relative KIR gene frequencies of three South African cohorts were investigated with the resultant compilation of a KIR database. DNA from selected samples was amplified using a sequence specific primer-polymerase chain reaction (SSP-PCR) method. This process involved the use of specific primers for identifying 17 currently known KIR genes, of which 2 are non-functional pseudo-genes.

The PCR products were loaded onto agarose gel, and electrophoresed before visualising on an ultraviolet (UV) trans-illuminator, in order to determine the presence or absence of the genes in question. Once the genes had been identified, a database was created in order to determine the frequency of the particular genes. Analysis included the identification of new haplotypes and mutations.

For each sample the presence or absence of each gene locus was reported. The gene and haplotype frequency as well as the KIR locus frequency (KLF) and linkage disequilibrium (LD) were established for each population. χ^2 (Chi^2) tests were used to test the "null" hypothesis for KIR two-locus association within each population cohort.

The aim of the project is to better understand the role of KIR in immunity. Information gathered from the study may be used in a possible PhD to determine further genetic linkages. The results may contribute to the understanding of HIV/AIDS progression and treatment.

CHAPTER 2

Literature Review

2.1 NK cells

Natural Killer cells make up 10 to 15 % of circulating leukocytes and play a pivotal role in innate immunity (Ljunggren *et al.* 1990). As their name indicates, NK cells are functionally identified by their ability to kill certain tumour and virally infected cells. Without the need for prior stimulation, NK cells fill the gap between the onset of illness and humoral and cell specific immune response (Bancroft *et al.* 1993, Trinchieri *et al.* 1989). Down-regulation of HLA expression in virus-infected cells renders these cells resistant to cytotoxic T-lymphocytes (CTLs) lysis. However, aberrant levels of HLA predispose NK cell lysis and as such both T-cells and NK cells represent complementary arms of the cellular immune response.

Derived from the lymphoid progenitor cells, NK cells have a large cytoplasm with granules and a bean shape eccentric nucleus. All developmental stages are completed in the bone marrow under the influence of stimulating factors from stromal cells (SC) providing growth signals for NK cells (Colucci *et al.* 2003). Important factors for NK cell development include interferon-γ (IFN-γ), which is produced by stromal cells in the presence of interleukin-2 (IL-2) (Scalzo *et al.* 2002). Produced by dendritic cells, IL-15 is also important, not only for NK cell development, but for the survival and homeostasis of mature NK cells (Piccioli *et al.* 2002, Jamieson *et al.* 2004).

Once released from the bone marrow, NK cells travel through the body in a partially activated state, using this as a method for self-tolerance while still possessing the ability to respond quickly to a viral infection (Pobezinskii *et al.* 2005). NK cells establish themselves in the spleen, lymph nodes and in the peripheral blood, where up-regulation by a factor of 20 to 100 fold helps to mediate inflammatory responses (Pobezinskii *et al.* 2005). Soluble factors which are important for this up-regulation include IL-12, IL-18 and IFN-γ, all of which are produced by dendritic and stromal cells (Ferlazzo *et al.* 2004, Gerosa *et al.* 2002, Perussia *et al.* 1996). At a site of

infection NK cells are transported across vessel barriers through a process known as "diapedesis" (Kitayama *et al.* 1993). Surface-receptors on NK cells interact with chemokines released from neutrophils and macrophages attracting NK cells to the site of infection (Loetsher *et al.* 1996).

2.2 NK cell receptors

NK cells express a range of receptors, which aid in their functional activity and regulation. Lacking the expression of cluster of differentiation 3 (CD3) and CD4, NK cells possess two main types of HLA Class I specific receptors. The first of these include the immunoglobulin-superfamily (ig-sf) receptors which mediates the killing of all viruses as well as tumour cells (Biron *et al.* 1999). C-type lectin receptor is the other main type of receptor, and is involved in the regulation of the adaptive and innate immune responses through the release of chemokines and cytokines (Biron *et al.* 1999).

The ig-sf receptors include KIR and leukocyte immunoglobulin-like receptor family (LILR). These HLA specific receptors are located on chromosome 19 as part of the LRC and stretches over a total DNA length of approximately 1 mega base (Mb) encompassing over 25 genes, as depicted in Figure 2.1, page 10.

The second family of receptors, the C-type lectin receptors, include the CD94/NKG2 heterodimer, and is located centromeric to the natural killer complex (NKC) on chromosome 12 (Barten *et al.* 2001). Along with these two main types of receptors, approximately half of the human NK cell repertoire expresses CD8 molecules which functions as a co-receptor for HLA Class I association (Seaman *et al.* 2000).

2.3 Leukocyte immunoglobulin-like receptors

LILR which was previously known as immunoglobulin like transcript (ILT) and leukocyte inhibitory receptor (LIR) has been proposed as being an ancestor to KIR (Cosman *et al.* 1997, Samaridis *et al.* 1997). While possessing both inhibitory and

activating activities LILR receptors also interacts with HLA Class I molecules (Fanger *et al.* 1999, Nakajima *et al.* 1999). As depicted in Figure 2.1 page 10, LILR is located centromeric to KIR, where the two regions (comprised of six and five loci each) face opposite transcriptional direction with leukocyte-associated inhibitory receptor (LAIR) separating the two loci (Canavez *et al.* 2001, Wende *et al.* 2000).

Leukocyte imunoglobulin-like receptor has either two or four extracellular domains with either a short or long cytoplasmic tail (Vivier *et al.* 1997). Like KIR, LILR with short cytoplasmic domains can associate with molecules containing ITAMs and contribute to cell activation. Furthermore, long cytoplasmic domain LILR contains four ITIMs which allow these receptors to inhibit cell lysis in much the same way KIR does (Vivier *et al.* 1997).

2.4 CD94/NKG2 receptors

Located on chromosome 12 the C-type lectin receptor complex is part of the natural killer complex and is made up of a single non-polymorphic CD94 gene as well as NKG2 genes 1-5. These are named A/C/D/E/F where B (not shown in Figure 2.1) is a non-functional alternative splice variant of A (Adamkiewicz *et al.* 1994, Houchins *et al.* 1991, Sobanov *et al.* 1999).

NKG2 receptors present on NK cells, bind to HLA complexes only when forming disulfide-linked hetero-dimers creating CD94/NKG2 receptors (Brooks *et al.* 1997, Carretero *et al.* 1997). CD94 functions as a stabilising molecule and lacks intracellular signalling motifs.

Inhibitory NKG2A exhibits a long cytoplasmic tail which contains ITIMs and upon tyrosine phosphorylation recruits Src homology-containing tyrosine phosphatases (SHP). Activating receptors on the other hand interact with adaptor proteins for signal transduction due to their short cytoplasmic tails in much the same way KIR activating receptors do.

The ligand for receptors CD94/NKG2A/C/E is the non-classical major histocompatibility complex (MHC) Class Ib molecule, HLA-E (Braud *et al.* 1998, Lee *et al.* 1998). HLA-E peptides are derived from the leader sequence of subset HLA Class I molecules (Borrego *et al.* 2002). Loss of MHC Class I expression leads to a reduction in cell surface HLA-E, rendering the cell susceptible to NK cell mediated cytolysis (Leong *et al.* 1998). The activating 2C, 2E receptors compete with the inhibiting 2A receptors for binding superiority. The affinity of inhibitory receptor 2A is stronger towards HLA-E than that of activating receptor 2C, and as such HLA-E/2A provides protection for targeted cells (Brooks *et al.* 2000, Vales-Gomez *et al.* 1999).

NKG2D interacts with the two variable ligands, macrophage inhibitory cytokine (MIC) MIC-A, MIC-B and the human cytomegalovirus glycoprotein (UL16) (Cosman *et al.* 2001). In situations of cell "stress" or neoplastic changes, these ligands are up-regulated and evade the immune response. NKG2D recognises these ligands and provides alternative immune protection (Barham 2000, Sutherland *et al.* 2001).

2.5 CD56 receptors

One of the receptors NK cells use for functional activity includes CD56, which is an iso-form of the neural-cell adhesion molecules (N-CAM) (Lanier *et al.* 1991). While the CD56 receptor function remains unclear, the concentration expressed on NK cells divides these receptors into two subtypes. CD56 expressed in low concentrations on NK cells ($CD56^{dim}$) are associated with active cytotoxic activity due to the relative high level of KIR and other surface markers expressed.

High levels of CD56 ($CD56^{bright}$) expression is associated with cytokine production which include IFN-γ, tumour necrosis factor-α and -β (TNF-α/-β) and interleukin-10/-13 (Cooper *et al.* 2001). While 90 % of NK cells express low-density CD56 ($CD56^{dim}$) the remaining 10 % express high-density CD56 ($CD56^{bright}$) and functions in immune regulation after being stimulated. Being able to respond quickly to IL-2, $CD56^{bright}$ expression is up-regulated showing similar levels of cytotoxicity as their counterpart ($CD56^{dim}$) (Robertson *et al.* 1992).

While possessing lytic granules it has also been proven that immature CD56 ($CD56^{dim}$) NK cells use tumour necrosis factor-related apoptosis-induced ligands (TRAIL), rather than releasing perforin and granzyme, as is the case in mature CD56 ($CD56^{bright}$) cells (Loza *et al.* 2001).

2.6 KIR receptors

Killer-cell immunoglobulin-like receptors was first described by Harel-Bellan *et al.* (1986) and was initially known as "killer inhibitory receptor". As part of the immunoglobulin-superfamily they were described as having a central role in innate immunity by stimulating target cell apoptosis through cytotoxic effects (Ljunggren *et al.* 1990, Moretta *et al.* 1990).

The names given to KIR genes are represented by the structure of the molecule they encode, these being either 2 or 3 extracellular domains (KIR2D, KIR3D), with variable cytoplasmic characteristics, "S" for short and "L" for long cytoplasmic tails (Marsh *et al.* 2003). While short (KIR2DS, KIR3DS) cytoplasmic tails interact with activating receptors, long (KIR2DL, KIR3DL) cytoplasmic tails transduce inhibitory signals and contain one or more ITIMs (Lanier *et al.* 1998).

Agreement was reached by the HUGO Genome Nomenclature Committee (HGNC) that a total of seventeen genes are recognised (Marsh *et al.* 2003). Fifteen of these genes are functional, with two pseudo-genes (2DP1, 3DP1) translating into non-functional products (Wilson *et al.* 2000). Table 2.1 lists the various gene names, descriptive name and number of alleles allocated to each KIR gene (Marsh *et al.* 2003).

Table 2.1 KIR gene names, descriptive names, submitting author and number of alleles (Marsh *et al*. 2003)

Gene Symbol	Description	No of Alleles	Submitting author
KIR2DL1	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, number 1	10	Colonna *et al.* 1995, Wagtmann *et al.* 1995
KIR2DL2	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, number 2	5	Colonna *et al.* 1995, Wagtmann *et al.* 1995
KIR2DL3	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, number 3	10	Colonna *et al.* 1995, Wagtmann *et al.* 1995
KIR2DL4	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, number 4	18	Selvakumar *et al.* 1996
KIR2DL5A	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, number 5A	-	Vilches *et al.* 2000b
KIR2DL5B	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, number 5B	-	Vilches *et al.* 2000b
KIR2DS1	Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, number 1	4	Biassoni *et al.* 1996
KIR2DS2	Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, number 2	8	Colonna *et al.* 1995, Wagtmann *et al.* 1995
KIR2DS3	Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, number 3	3	Dohring *et al.* 1996
KIR2DS4	Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, number 4	9	Wagtmann *et al.* 1995, Dohring *et al.* 1996
KIR2DS5	Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, number 5	3	Dohring *et al.* 1996
KIR2DP1	Killer cell immunoglobulin-like receptor, two domains, pseudo-gene, number 1	-	Vilches *et al.* 2000b
KIR3DL1	Killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, number 1	22	Colonna *et al.* 1995
KIR3DL2	Killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, number 2	20	Colonna *et al.* 1995
KIR3DL3	Killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, number 3	7	Torkar *et al.* 1998
KIR3DS1	Killer cell immunoglobulin-like receptor, three domains, short cytoplasmic tail, number 1	6	Dohring *et al.* 1996
KIR3DP1	Killer cell immunoglobulin-like receptor, two domains, pseudo-gene, number 1	4	Vilches *et al.* 2000b

2.6.1 KIR genetics

The KIR locus is both polymorphic and highly homologous and is located on the short arm of chromosome 19 (19q13.4) as part of the leucocyte receptor complex (Trowsdale *et al.* 2001). The probability of two individuals inheriting the same KIR genotype (KG) is slim, with expression varying clonally, adding yet another layer of complexity. Arrayed in a head-to-tail fashion, KIR genes stretch over a 150 kb domain of DNA with each gene being approximately 10 to16 kb in length as depicted in Figure 2.1, page 10 (Uhrberg *et al.* 1997). Separation between all loci approximates a 2 kb stretch of DNA with the exception of a 14 kb sequence upstream from 2DS4 (Wilson *et al.* 2000).

Once a NK cell has committed to expressing a particular combination of KIR genes that pattern remains stable through time and cell division (Farag *et al.* 2003). Different combinations of expressed receptors as well as different clonal number variations combine to form the heterogeneous repertoire (Kubota *et al.* 1999). Expression of receptors does not seem to be random, with the entire KIR genotype being expressed selectively on all NK cells (Shilling *et al.* 2002). Diversity at the locus may be the result of selection pressure and as such has been proposed to mimic HLA loci drift.

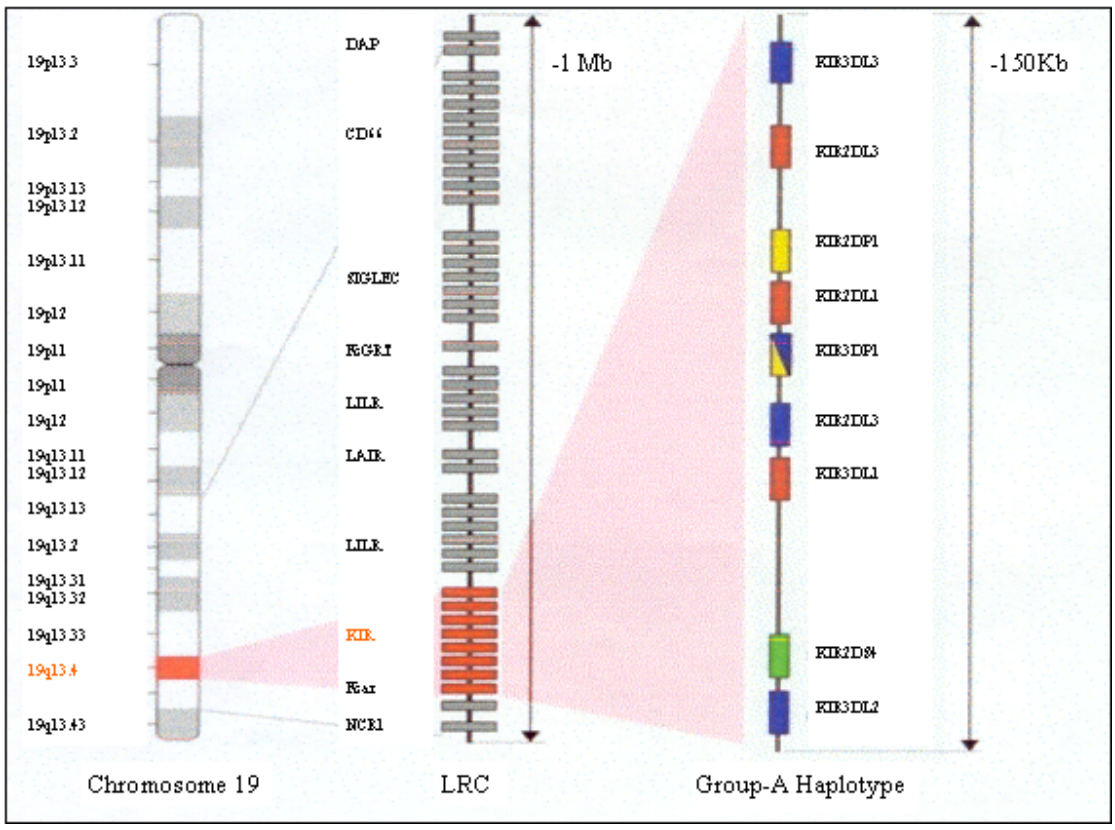

Figure 2.1 KIR genes located within the leukocyte receptor complex (Figure was modified from European Bioinformatics Institute: KIR Database)

2.6.2 KIR receptor structure

KIR2D receptors exist in two possible variants, type I and type II. The first of these variants type I 2D receptors, includes the pseudo-gene KIR2DP1, as well as KIR2DL 1-3 and KIR2DS 1-5. These receptors are missing the D0 and as a result only express the D1 and D2 protein structures as depicted in Figure 2.2, page 11 (Vilches *et al.* 2000c).

Figure 2.2 depicts type II KIR2D receptors which include KIR2DL4, KIR2DL5A and KIR2DL5B. Type II KIR2D lack the D1 domain and as such only express D0 and D2 (Selvakumar *et al.* 1997). Expression of the membrane-distal ig-like domain in type II receptors, resemble the D0 present in KIR3D receptors (Vilches *et al.* 2004).

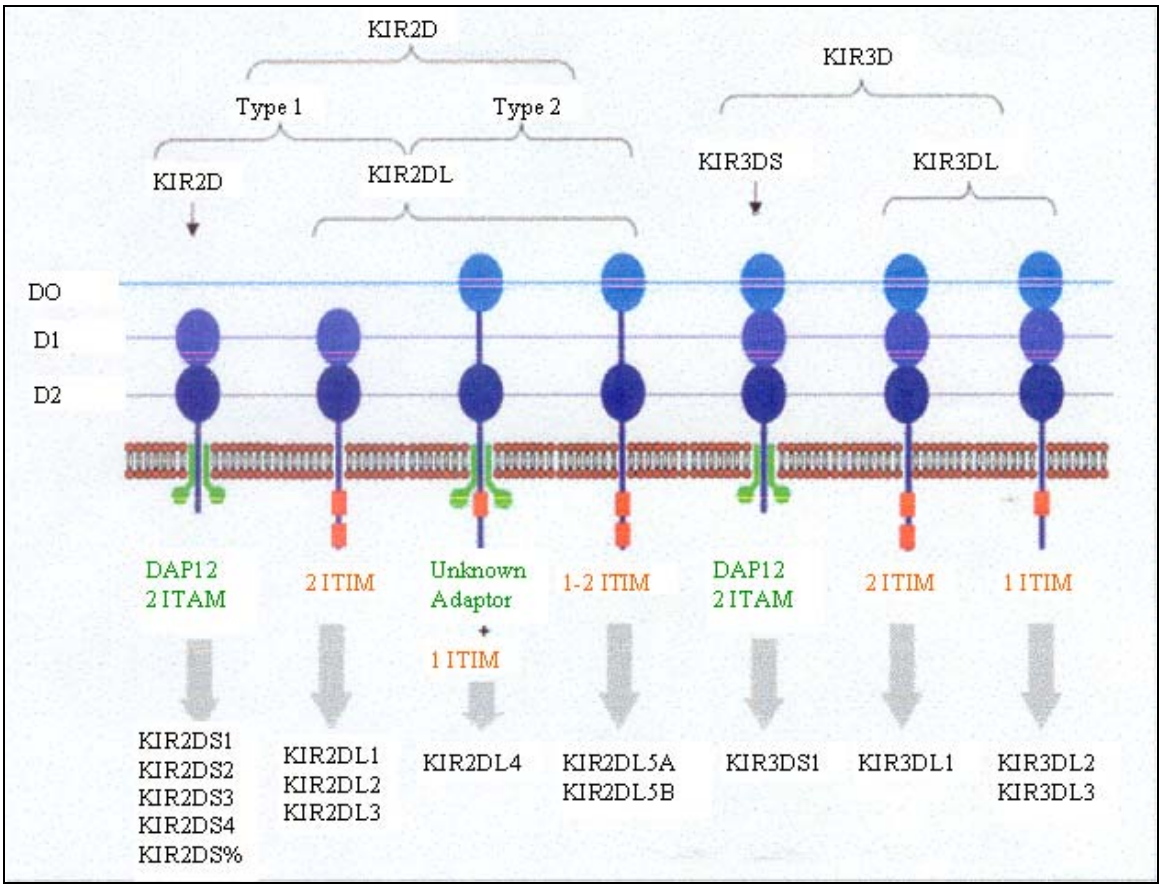

Figure 2.2 Domain structure of the KIR molecules (Figure was modified from European Bioinformatics Institute: KIR Database)

Figure 2.2, depicts KIR3D receptors, KIR3DL1, KIR3DS1 KIR3DL2 and KIR3DL3 all possessing D0, D1 and D2 domains. With only one of the 3D receptors possessing a short cytoplasmic tail, the remaining 3DL1-3 all contain long intracellular tails.

Structural folding of only three KIR family members has been described here, these include KIR2DL1, KIR2DL2 and KIR2DL3 as can be noted in Figure 2.3, page 12 (Sawicki *et al.* 2001). D1 and D2 are made up of 102 amino acids and 98 amino acids respectively and contain 40 % sequence similarity, suggesting domain duplication (Boyington *et al.* 2000). While the first domain is folded towards the cell surface, the exposed junction between the two domains allow for the ligand-binding region, which interacts with HLA molecules (Boyington *et al.* 2000).

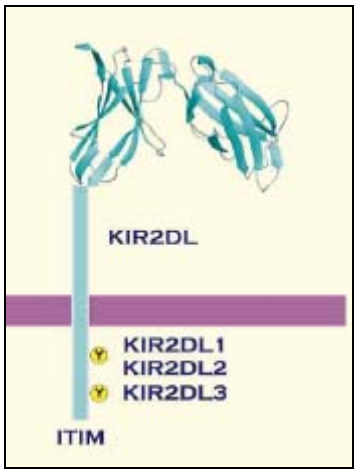

Figure 2.3 KIR structural folding (Figure was modified from Sawicki *et al.* 2001)
Y = ITIMs

This hinge region varies between receptors and is stabilized by a highly conserved inter-domain hydrophobic core consisting of leucine17, methionine69, valine100, isoleucine101, threonine102, histidine138, phenylalanine178, serine180, praline185, tyrosine186 and tryptophan188 (Chapman *et al.* 2003). Angles varying from 66^{o} in 2DL1 to 81^{o} in 2DL2 and 2DL3 have also been reported and are used as a tool to distinguish these receptors phenotypically (Boyington *et al.* 2000).

2.6.3 KIR EXON/INTRON arrangement

Exon/Intron arrangement is similar among all KIR genes with only limited variation from one gene to the next. Starting from the N-terminus (as depicted in Figure 2.4, page 14) protein structure is encoded by eight exons in 2D receptors and nine exons in 3D receptors which illustrates the similarities between these two receptors (Wilson *et al.* 1997).

Exons 1 and 2 form the signalling sequences, followed by exon 3 encoding the membrane distal domain while the middle and the proximal domains are encoded by exons 4 and 5 (Wilson *et al.* 1997). Exons 6 and 7 translate into the stem and the trans-membrane region in both inhibitory and activating receptors (Trowsdale *et al.* 2001). The final two exons encode the cytoplamic domain, with the number of amino

acids varying from 23 amino acids in 3DS1 receptors to 116 amino acids in 2LD4 receptors as depicted in Figure 2.4, page 14 (Wilson *et al.* 2000).

Type I 2D receptors, which transcribe from the 2DL1, 2DL2/3 and the 2DS genes, all contain exon 3, 4 and 5 with exon 3 being a pseudo-exon. Pseudo-gene 2DP1, which contains the same exons as type I 2D genes possesses a single base pair deletion in exon 4, which results in a frame shift and consequently producing a stop codon (Vilches *et al.* 2002).

Type II 2D proteins (2DL4, 2DL5A and 2DL5B), on the contrary, contain domains coded by gene exons 3 and 5 with exon 4 being absent in the protein structure. Exon 4 is spliced out due to a three base-pair deletion, with the resultant structure of domains D0 and D2 expressed, consequently missing the D1 domain (Selvakumar *et al.* 1997).

Due to a 1.5 kb deletion in 3D receptors which include KIR3DP1, only contain the one leader sequence (other receptors contain two, Figure 2.4) which ultimately results in the removal of exon 2. The remaining receptors all possess three functional exons (3-5) resulting in their classification as 3D receptors (Wilson *et al.* 2000). Additional non-functional gene fragments KIRC1, which resembles KIR3D structures are also found within the KIR locus. KIRC1 lacks the exon for the stem domain and as such no products are transcribed (receptor gene not shown).

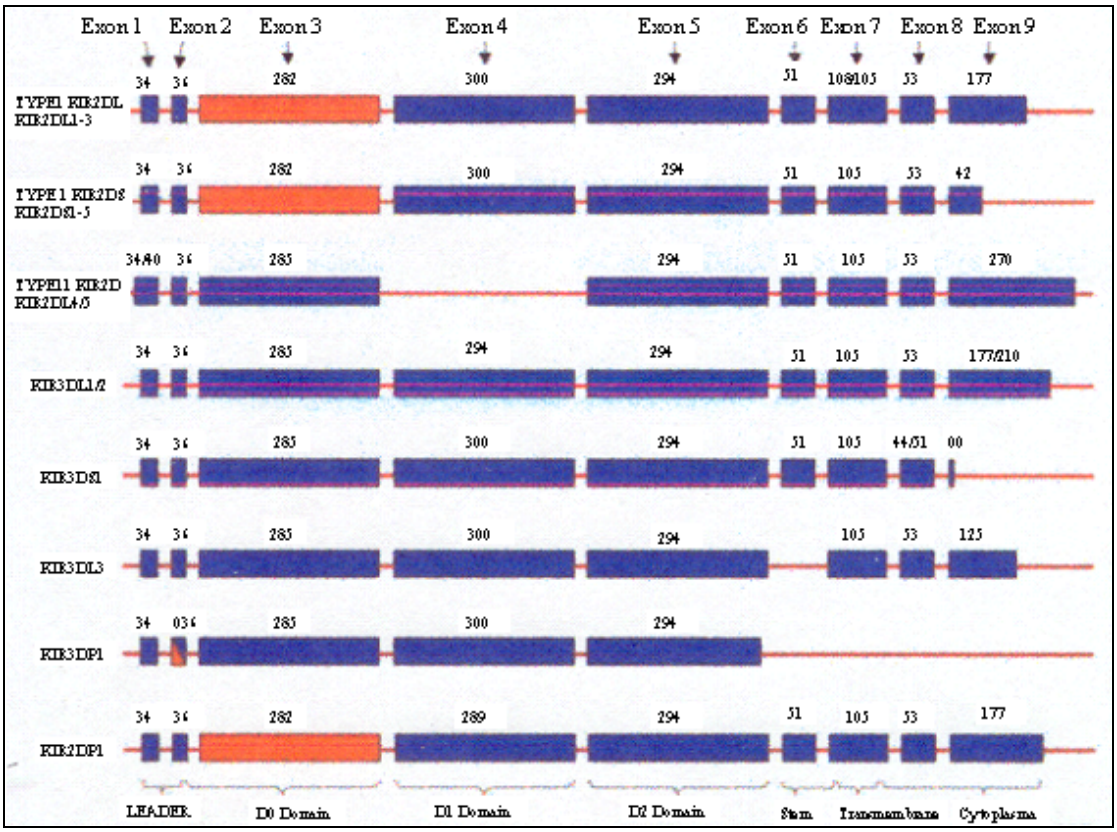

Figure 2.4 KIR genes arrangement (Figure was modified from European Bioinformatics Institute: KIR Database)

Orange block = p

2.6.4 KIR haplotypes

Extensive variation has been shown in the number and type of KIR genes present within individuals. Both the number and the organised arrangement of inhibitory and activating KIR genes determine the particular haplotype (Shilling *et al.* 2002). Over 100 different KIR haplotype profiles have been described thus far and this number keeps expanding as new haplotypes are discovered (Hsu *et al.* 2002b, Gómez-Lozano *et al.* 2002, Uhrberg *et al.* 2002).

All haplotypes are flanked by KIR3DL3 at the centromeric end and KIR3DL2 at the telomeric end as depicted in Figure 2.5 (Wilson *et al.* 1997). KIR3DP1 as well as the down stream gene KIR2DL4 are situated centrally, together making up the framework loci present in all individuals (Wilson *et al.* 2000). This configuration can be seen in Figure 2.5 where the black boxes represent the two pseudo-genes.

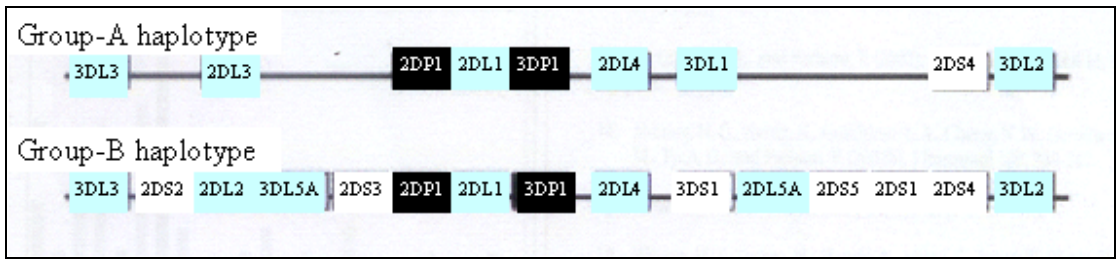

Figure 2.5 KIR A and B haplotype content (Figure was modified from Rajalingam 2003)

Dependent on the KIR genes present, two major haplotypes are formed, namely A and B (Wende *et al.* 1999). As depicted in Figure 2.5 the A-haplotype (AH) is relatively simple, conserved and consists of a fixed number of genes. B-haplotype (BH) on the contrary is expansive, comprising more activating KIR genes, with the gene content varying greatly from haplotype to haplotype (Shilling *et al.* 2002). Different criteria have been used for the assignment of the two haplotypes, while inclusion of 2DL1 and 2DL3 has been nominated A-haplotypes, the absence of both 2DL1 and 2DL3 which has been nominated B-haplotypes (Hsu *et al.* 2002a, Witt *et al.* 1999). Further inclusion of the C-haplotype involves the absence of 2DL1, 2DL2 and 2DL3 as first reported by Witt and co-workers (Norma *et al.* 2001, Witt *et al.* 1999)

The A-haplotype is more frequent in Caucasian and Japanese populations with a total frequency of 86 %, where homozygous individuals for the A-haplotype make up nearly 50 % of these populations (Yawata *et al.* 2002). In Australian Aborigines the B-haplotype dominates with a frequency of 90 % (Toneva *et al.* 2001). Included into the AH there are 9 genes, 6 inhibitory genes (depicted as blue boxes in Figure 2.5) as well as the 2 psuedo-genes (depicted as black boxes, in Figure 2.5), with only the one activating gene KIR2DS4 as depicted by the white box in Figure 2.5 (Maxwell *et al.* 2002).

Comparison between the A- and the B-haplotypes shows a far greater variety of subtypes within the B-haplotype. When comparing stimulating receptors between these two haplotypes, as indicated in Figure 2.5 the B-haplotype has an array of

activating receptors 2DS1, 2DS2, 2DS3, 2DS5, 3DS1 and 2DS4, compared to the A-haplotypes having only one activating receptor 2DS4.

Individuals homozygous for the A-haplotype inherit only the one activating receptor 2DS4, with a total of seven functional genes. Individuals homozygous for the B-haplotype, express 6 functional activating receptors with a total of 13 expressed receptors.

Although there are numerous people with no activating receptors in their haplotypes, no individual has been identified for which no inhibitory receptors are expressed (Thananchai *et al.* 2007). This is due to the fact that activating receptors are more dispensable than inhibitory receptors, where lack of inhibitory receptors would result in limited or no inhibition of cytotoxic effects (Burshtyn *et al.* 2003).

Linkage disequilibrium (LD) patterns of KIR loci have been studied for both the A- and B-haplotypes (Witt *et al.* 1999). No LD has been noted for the A-haplotype at the gene level but patterns have been noted between different alleles (Shilling *et al.* 2002). The B-haplotype on the other hand shows strong LD between many KIR genes demonstrating the continual drift within this haplotype (Witt *et al.* 1999).

2.6.5 Gene inheritance patterns

Certain inheritance patterns are due to unequal crossing over, and have been observed when comparing haplotypes from individual to individual (Michelmore *et al.* 1998). An example is where 3DL1 and 3DS1 are inherited alternatively, occupying the same position on different haplotypes (Michelmore *et al.* 1998). Further studies revealed that they are indeed alleles of a single locus as reported by Wilson and co-workers (Wilson *et al.* 2000, Gardiner *et al.* 2001). Individuals missing both 3DL1 and 3DS1 have been identified by Gardiner and co-workers, as well as individuals containing both genes, where these are rare they are derived from unequal crossover allowing for the inheritance of both loci together (Gardiner *et al.* 2001).

Wilson and co-workers proposed that 2DL2 may have originated due to a recombination event taking place between 2DL1 and 2DL3 (Wilson *et al.* 2000). Sequence similarity patterns of 2DL2 and 2DL3 indicate that they did undergo non-reciprocal recombination, explaining why they segregate as alleles of the same locus (Norman *et al.* 2001, Witt *et al.* 1999). The pseudo-gene 2DP1 which is located between 2DL1 and 2DL3 is lost during the crossing over event and is missing on a haplotype containing 2DL2, resulting in 2DL1 and 2DP1 being present or absent together (Wilson *et al.* 2000).

Alleles 2DS1 and 2DS4 are also inherited alternatively, with 2DS1 being absent when 2DS4 is present and *vice versa*. These inherited genes do not occupy the same locus, distinguishing themselves from previously mentioned inherited pairs, however they are located next to each other within the KIR haplotype (Norman *et al.* 2001, Wilson *et al.* 2000).

Williams and co-workers (2003) have shown the presence of individuals with three copies of 2DL4, 3DL1 and 3DS1 genes. This is the result of recombination events leading to genes being duplicated. Further analysis of 11 subjects revealed that the promoter region between genes 3DP1 and 2DL4 in 10 individuals share the same sequence (Williams *et al.* 2003).

2.6.6 KIR gene regulation

Specific cytokines, essential for the expression and development of KIR are yet to be defined (Miller *et al.* 2001). Miller and co-workers reported that bone marrow derived stromal cells are involved in the induction of KIR expression, and it has been proposed that contact-dependent signals are responsible for these actions (Miller *et al.* 2001).

The KIR repertoire can be achieved in the absence of cells expressing HLA Class I, indicating that positive or negative selection is not required from NK cell development (Belkin *et al.* 2003, Ugolini *et al.* 2001). The effect is that KIR is

expressed where there are no known HLA receptors. The opposite is true for HLA expression, where no KIR receptor is expressed (Gumperz *et al.* 1996).

Sharing 90 % DNA sequence similarity, KIR gene promoter regions are highly similar in the upstream sequence, suggesting a similar regulation method for all KIR genes (Trowsdale *et al.* 2001, Valiante *et al.* 1997b). Concentrated phosphodiester-linked cytosine and guanine pair (CpG) islands are observed surrounding the transcription initiation region in most KIR genes, with the exception of KIR2DL4 and KIR3DL3 (Santourlidis *et al.* 2002).

Chan *et al.* (2003) as well as Santourlidis *et al.* (2002) proposed a crucial role of DNA methylation as one method of gene regulation for KIR genes (as shown in Figure 2.6, page 19). Intrinsic conditions rather than external surroundings may dominate regulating patterns of KIR receptors (Chan *et al.* 2003, Santourlidis *et al.* 2002). Epigenetic mechanisms rather than differences in KIR promoters have been proposed to be essential for maintaining expression (Chan *et al.* 2003). Their proposal suggests that CpG islands located upstream of KIR genes, are targets of frequent modification, resulting in inhibition of transcription when DNA methylation occurs (Santourlidis *et al.* 2002).

Santourlidis and co-workers (2002) investigated the DNA methylation status of the KIR locus in NK cells, revealing that these CpG islands are demethylated in expressed KIR genes and highly methylated in silent KIR genes. Another equally important method of KIR gene regulation includes the regulation of histones (H) (Turner *et al.* 2002). The methylation, acetylation and phosphorylation of histone N-terminal amino acids regulate chromatin accessibility by transcription proteins, thus regulating activity through the folding and unfolding of a secondary regulator.

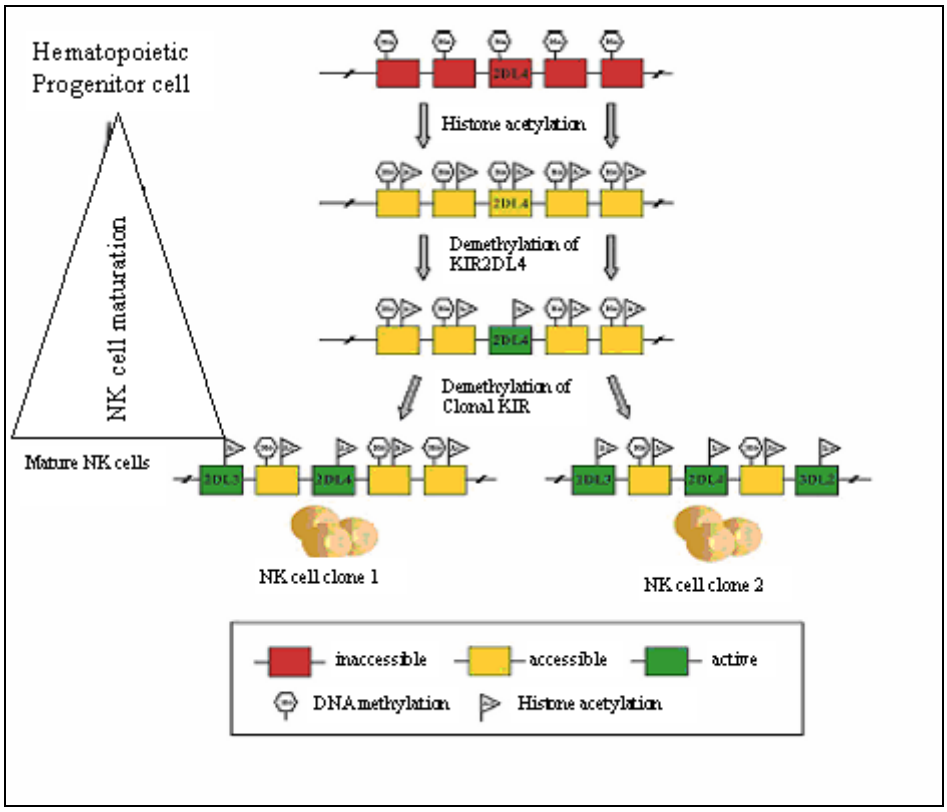

Figure 2.6 KIR gene regulation (Figure was modified from Uhrberg 2005)

Histone amino acids, which show important gene activation properties when acetylated, include markers such as H3 at the Lys9 position, as well as Lys14 and Lys8 on H4 (Jenuwein *et al.* 2001). This method of histone regulation together with DNA methylation provide increased control over KIR gene expression.

Uhrberg and co-workers proposed that KIR receptors travel through four epigenetic stages, from haematopoietic stem cells to mature NK cells as depicted in Figure 2.6 (Uhrberg 2005). The first of these stages include the silencing of all KIR genes through chromatin condensation as well as DNA methylation. Following KIR silencing, histones acetylation occurs with a more open chromatin structure as maturation proceeds (Uhrberg 2005). Demethylation of KIR genes is initiated with KIR2DL4 as seen in Figure 2.6 where this receptor is driven by a distinct promoter allowing increased expression potential (Stewart *et al.* 2003). The final developmental stage includes the demethylation of the remaining KIR gene and the resultant KIR repertoire as seen in Figure 2.6. Within this selection process no bias is shown due to

the relative similarities of the promoter regions resulting in non-specific selection and the creation of maximum diversity between clones (Uhrberg 2005).

During selection, self-tolerance may be ensured via a sequential pattern of expressed KIR, suggesting a type of selection to form the NK repertoire (Young *et al.* 2001, Miller *et al.* 2001). At present there appears to be no selection mechanisms for KIR during the maturation process of NK clones, as is the case in T-cell receptors (TCR) (Young *et al.* 1998).

2.6.7 KIR signalling

Apart from one exception (2DL4), discussed at the end of this section, page 23, all KIR receptors fall within two broad categories dependent on whether they are inhibitory receptors or activating receptors, resulting in complimentary receptor action (Uhrberg *et al.* 2002). Interaction with the target cell generates opposing inhibitory or activating signals depending on which receptors binds to HLA, as depicted in Figure 2.7, page 21.

Two possible outcomes are considered with respect to target cell interaction through the KIR/HLA molecules. The first being “auto tolerant”, while the second is known as “auto aggression” whereby target cells are programmed for cell death (Hsu *et al.* 2002b). Of the latter there are a further two possible reasons for the induced “auto aggression”, the first being a consequence of “missing self” due to reduced HLA expression. The second is attributed to “missing ligand” where abnormal or alternate ligands are expressed in the HLA binding groove (Shimizu *et al.* 1989, Ljunggren *et al.* 1990).

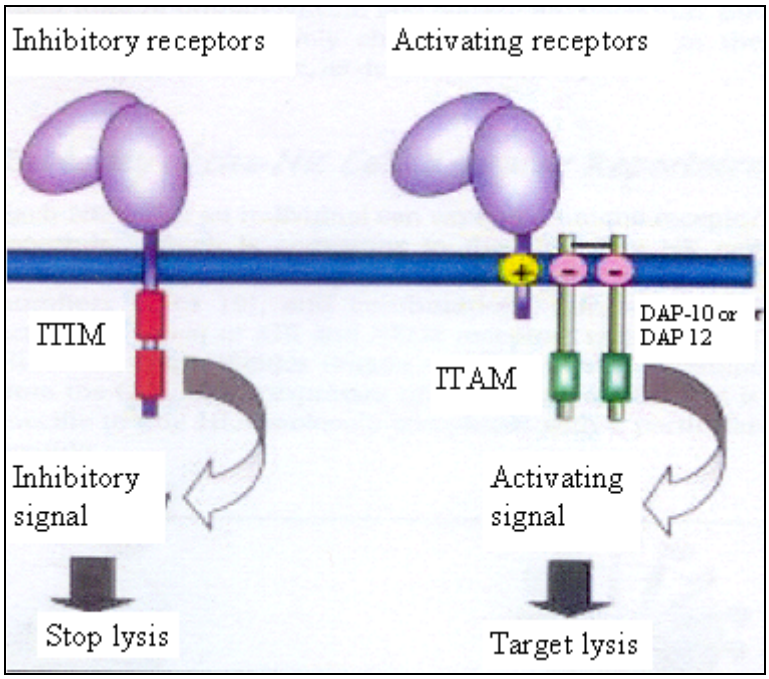

Figure 2.7 Activating and inhibiting intracellular signals (Figure was modified from Rajalingam 2003)

A single KIR/HLA interaction does not determine cell destruction, but rather a "net balance" response resulting in either lysis, or inhibition of target lysis. In particular circumstances, inhibitory signals can override activatory signals as a result of higher binding affinity of the inhibitory receptors, thus providing increased protection against "auto-aggression" (Biassoni *et al.* 1997, Vales-Gomez *et al.* 1998).

Activating receptors on their own contain short cytoplasmic domains with no signalling function as depicted in Figure 2.7. Trans-membrane (TM) domains of activating receptors interact with intracellular signalling proteins enabling activated cellular responses and target lysis.

Both *DAP10* and *DAP12* genes are located centromeric to the LRC on chromosome 19 and their products function as a signalling protein through their TM interactions with activating receptors. These TM interactions are made possible by basic amino acids, such as aspartic acid, interacting with acidic amino acids within the TM region of activating receptors. As depicted in Figure 2.7 the TM interactions form salt bridges that associate the receptor with the signalling proteins (Lanier *et al.* 2000, Lanier *et al.* 1998).

The ITAM domains contain a (Y x x [L/V] x [6-8] Y x x L/V) repeat, valine (V) or luecine (L) is sub-repeated 6-8 times within one ITAM. X represents any amino acid and plays no functional role within the signalling process (Futterer *et al.* 1998). ITAMs encoded within *DAP10* or *DAP12* proteins allow for the trans-phosphorylation of the tyrosine through the signalling process, once receptor clustering has occurred (Maxwell *et al.* 1999). Phosphorylated ITAMs provide docking sites for zeta chain-associated protein 70 kilo Dalton (ZAP70) and spleen tyrosine kinase (Syk)-family tyrosine kinases via the Src homology 2 (SH2) domain, both of which are expressed by all NK cells (Leibson *et al.* 1997, Brumbaugh *et al.* 1997). Kinases activated by ITAMs lead to recruitment and activation of downstream elements such as phospholipase C-γ (PLC- γ) and growth factor receptor bound 2 (Grb2). Both of these cascade proteins play a pivotal role in the release of stored calcium, ultimately resulting in polarisation and extocytosis of granules containg lytic enzymes (Maxwell *et al.* 1999).

Inhibitory receptors, on the contrary, communicate through ITIM domains located within the cytoplasmic tail. Most inhibitory KIR possesses two ITIM domains, although one or more than two have been reported (Bruhns *et al.* 1999). ITIMs are made up of the protein sequence (I/V x Y x x L) where hydrophobic iso-leucine/valine (I/V) is positioned one residue upstream of a tyrosine (Y) (Butcher *et al.* 1998). A further leucine (L) is located two residues downstream of the tyrosine where x represents any amino acid. This pattern is then repeated within the cytoplasmic domain depending on the number of ITIMs present (Tomasello *et al.* 1998).

Once receptor clustering occurs, phosphorylation of the tyrosine residues within cytoplasmic tail ITIMs generates signals which polarize cytolytic granules for exo-cocytosis (Fomina *et al.* 1995). ITIM tyrosine also becomes phosphorylated within the receptor cluster before engaging phosphatases to counteract cellular activation (Burshtyn *et al.* 1996). Phosphorylated tyrosine then binds and activates SHP-1 and SHP-2 through their SH2 domains. This prevents the phosphorylation cascade via the removal of any phosphate added during activation within the receptor cluster, ultimately leading to the inhibition of NK cell activation (Campbell *et al.* 1996). SH-

containing inositol 5-phosphatase (SHIP) (an alternative to SHP-1 -2) also inhibits activated tyrosine ITAMs, depending on the method of activation used within the cascade (Wang *et al.* 2002)

One exception is the KIR2DL4 receptor, which contains one ITIM within its cytoplasmic tail, and as such has an inhibitory effect (Rajagopalan *et al.* 1999, Canton *et al.* 1998). KIR2DL4 also contains a positively charged arginine residue in its trans-membrane region which facilitates the interaction with adaptor protein *DAP10* (Faure *et al.* 2002, Yusa *et al.* 2002). Thus it has been reported that 2DL4 carries both inhibitory as well as activating functions depending onto which HLA it binds (Faure *et al.* 2002, Yusa *et al.* 2002).

2.6.8 KIR and disease

2.6.8.1 HIV

Because of the recent discovery of KIR, a limited number of studies have been undertaken about KIR disease association. Playing an essential role in the protection against viral infections and tumours, KIR/HLA receptor-ligand combinations appear to play a significant role in certain diseases. The synergistic relationship between these polymorphic loci ultimately regulates NK cell mediated immunity against infectious agents and are thus predisposing genetic factors to status of health. Martin *et al.* described delayed progression of HIV to AIDS, in individuals possessing HLA-B antigens with iso-leucine at position 80, when interaction occurs with the 3DS1 receptor (Martin *et al.* 2002b). Patients without iso-leucine at position 80 on the HLA-B antigen experienced a rapid progression to AIDS (Martin *et al.* 2002b).

2.6.8.2 Autoimmunity

Natural selection target the genes associated with developed resistance and thus predispose these genes for failed resistance when further selection occurs. Polarisation of diversity towards one KIR haplotype might confer protection against one disease, while another less deadly disease leads to increased mortality. Autoimmune diseases are one example where increased risk is developed when predisposing KIR genes are present. Plausible causes to autoimmune disease states can be attributed to activating KIR molecules undergoing clonal expansion in the absence of effective inhibitory

receptors as stated previously (Namekawa *et al.* 2000). Yen *et al* (2001) and Namekawa *et al* (2000) reported that patients with the 2DS2 receptor showed an increased incidence of rheumatoid arthritis compared to rheumatoid vasculitis. Further autoimmune studies showed increased susceptibility towards developing psoriatic arthritis amongst individuals with 2DS1 or 2DS2 in combination with missing receptors 2DL1, 2DL2 and 2DL3 (Martin *et al.* 2002a).

2.6.8.3 Foetal rejection

Interactions between KIR2DL4 and HLA-G on human trophoblasts, has been describe as providing protection against maternal NK cell mediated rejection of hemi-allogeneic foetal cells (Witt *et al.* 2000). Deletion of exon 6 during messenger ribonucleic acid (mRNA) processing results in the formation of several 2DL4 allele variants with functional properties. 2DL4 alleles may vary in their ability to control the rejection of foetal cells as observed in transplantation (Witt *et al.* 2000).

In transplantation constructive hematopoietic transplants are possible where donor NK cell allo-reactivity is capable of preventing leukaemia relapse within recipient patients, while also protecting against graft versus host disease (GvHD) (Ruggeri *et al.* 1999, Ruggeri *et al.* 2002)

Development of new techniques and efficient typing systems will boost disease association studies, just as the HLA loci have been addressed in the past. New discoveries and better understanding regarding KIR in autoimmunity and transplantation will make therapeutic procedures possible, in order to overcome these ailments.

2.6.9 KIR evolutionary diversity

Haplotypes and sequences within and across KIR are evolving rapidly, possibly co-evolving with pathogens in order to mount suitable immune responses (Guethlein *et al.* 2002, Khakoo *et al.* 2000). It has been demonstrated that the human KIR gene structure and sequence are changing at a rate comparable or greater than that observed for HLA genes. This will lead to greater divergence between species at the KIR locus

(Gumperz *et al.* 1995). Further selective pressure may also be directed at KIR genes during early phases of infection, due to selection for variants which enhance innate immune responsiveness, thus increasing the rate of evolution and ultimately surpassing that of HLA (Martin *et al.* 2000). Continued expansion and evolution of KIR is required for the ability to interact with the changing HLA Class I molecules in a beneficial manner, thus complementing each other (Valiante *et al.* 1997a).

It was initially thought that KIR only occurred in higher primates, ungulates and other mammals (Mager *et al.* 2001). In chimpanzees, the closest living species to humans, ten KIR genes have been identified of which three appear to be orthologues of human KIR (Khakoo *et al.* 2000). One example of evolutional similarity between humans and other primates is the maintenance of KIR2DL4 which has persisted over millions of years (Fan *et al.* 2001). Similarities are so high that receptors between these species can recognise each other's HLA and mount a suitable response.

Chimpanzees and humans diverged approximately 5 million years ago, resulting in 98.4 % sequence similarity when comparing the two genomes (Sibley *et al.* 1990). This becomes evident when comparing the number and type of cytoplasmic KIR domains between humans and chimpanzees. KIR in humans has a virtually equal number of short and long cytoplasmic domains when compared to the predominantly long cytoplasmic domains present in chimpanzees (Khakoo *et al.* 2000). However, humans contain eight KIR genes with the D1/D2 domain configuration being in contrast to two D1/D2 domains present within chimpanzees. Chimpanzees contain six D0/D1/D2 genes, where humans only possess three of the D0/D1/D2 configuration genes (Fan *et al.* 2001). It can be speculated that the 3D receptors within chimpanzees have not lost any domains in order to evolve a receptor that is equivalent to the 2D human receptors.

Located on a DNA segment that is undergoing expansion and contraction over time, KIR have resulted in unequal crossing over and the duplication of certain genes. An example is that of the 2DL5 genes where two possible variants exist (Vilches *et al.* 2000a, Wilson *et al.* 2000). The first of these, KIR2DL5A possesses two ITIMs and is located at the telomeric region of the KIR locus. Located at the centromeric KIR region is KIR2DL5B which only possess one to two ITIM in its cytoplasmic domain.

Although these receptors share >99 % sequence similarity they are still grouped as two separate receptors, and as such four receptor variants are possible (Gómez-Lozano *et al.* 2002, Vilches *et al.* 2000a).

2.7 HLA

Located on the short arm of chromosome 6 the major histocompatibility complex stretches over 4 centimorgans of DNA containing more than 200 genes. These include HLA genes, which encode for the HLA molecules expressed on all nucleated cells. These molecules function in the binding and displaying of peptide fragments in order to be recognised by the relevant affector cells (Zinkernagen *et al.* 1974, Bhorkman *et al.* 1987, De Kock *et al.* 1997).

HLA Class I molecules have three α-chain genes, nominated human leukocyte antigen-A (HLA-A), human leukocyte antigen-B (HLA-B) and human leukocyte antigen-C (HLA-C), all of which encode for three domain molecules (α_1, α_2, α_3). β_2-microglobulin (β_2-M), transcribed by the β_2-microglobulin gene, associate with HLA Class I and acts as a stabilising molecule for the α-chain before expressing ligands on the cell surface (Snary *et al.* 1977).

HLA Class II on the other hand also consists of three variable receptors (HLA-DR, -DP and -DQ), all of which contains an α- and a β-chain. Included with these three expressed receptors, two variable HLA-DR molecules are possible. This is due to an extra β-chain, whose product can pair with the α-chain creating –DR$\alpha\beta_1$ or –DR$\alpha\beta_2$ depending on which β-chain binds. (Brown *et al.* 1993)

While HLA Class I peptides originate from the cytosolic compartments, Class II peptides are obtained from phagocytic vesicles that contains endocytosed and de-gradated pathogens. As well as presenting peptides from different compartments, peptides vary in size from 8 to 9 amino acids on HLA Class I molecules to 13 to 17 amino acid on HLA Class II molecules. All MHC molecules present peptides to NK as well as CTL cells. Each molecule represents a different range of peptides, affecting recognition and response in monitoring cellular status (Malnati *et al.* 1995). (Bjorkman *et al.* 1987).

In addition to the classic HLA Class I and II molecules, many other Class I type genes are expressed. These molecules are less polymorphic than the classical HLA Class I molecules, but are still functional to present antigen. They have been termed HLA Class Ib genes and like HLA Class I, associate with β_2-M when expressed on the cell surface (Crisa *et al.* 1997).

2.7.1 KIR/HLA recognition

Several KIR/HLA interactions have been described and is noted in Table 2.2 where HLA Class I sub-types have been indicated for each of the KIR/HLA gene interactions. Receptors 2DL1 and 2DS1 recognise the C2 epitopes as proposed by Colonna and co-workers (Colonna *et al.* 1992). As noted in Table 2.2, C2 epitopes include HLA-C*02, C*04, C*05, C*06, C*17 and C*18 all containing Asp at residue 77 and Lys at residue 80 (Colonna *et al.* 1992). Alternately receptors 2DL2/3 and 2DS2 recognise ligands from the C1 epitopes (C*01, C*03, C*07, C*08, C*13 and C*14), where these epitopes possess amino acids Ser and Asp at residues 77 and 80 respectively (Colonna *et al.* 1992, Winter *et al.* 1997).

Table 2.2 KIR interactions with their respective HLA molecules (Carrington and Norman 2003)

KIR2DL1 and KIR2DS1	KIR2DL2/3 and KIR2DS2	KIR3DL1/S1	KIR3DL2	KIR2DL4	KIR2DS4
Asn77/Lys80	Ser77/Asn80				
HLA-C (C2)	HLA-C (C1)	HLA-B Bw4	HLA-A	HLA-G	
C*02	C*01	B*08	A*03		C*04
C*04	C*03	B*13	A*11		
C*05	C*07	B*27			
C*06	C*08	B*44			
C*17	C*13	B*51			
C*18	C*14	B*52			
		B*53			
		B*57			
		B*58			

Receptors 3DL1 and 3DS1 interact with HLA-Bw4 and their respective ligands (Gumperz *et al.* 1995). Epitopes of the Class I HLA-B molecules comprise B*08, B*13, B*27, B*44, B*51, B*52, B*53, B*57 and B*58 as noted in Table 2.2.

KIR2DL4 binds to HLA-G and their interaction protects the foetus against maternal NK and CTL cell response, thus protecting against rejection of the foetus (McMaster *et al.* 1995). Ligand Bw6 (not shown) has as yet no known receptors and is distinguished from Bw4 by polymorphic alterations at residues 77 and 80 (Rajagopalan *et al.* 1999, Ponte *et al.* 1999). Ligands for receptors 2DL5, 2DS3, 2DS5 and 3DL3 remain as yet unknown (Vilches *et al.* 2000b).

2.7.2 KIR/HLA interaction

Interactions between KIR and HLA play an important part in immune surveillance, not only for signalling but also in adhesion and structural interaction. This is evident when observing arrangement between NK cells receptors and HLA on the target cells when receptor interaction occurs. Snyder and co-workers proposed that receptor clumping functions in both these tasks allow for increased signalling potential and increased signalling adhesion, as described for 2DL2/HLA-C (Snyder *et al.* 1999). KIR/HLA interaction patterns are not only clustered at the interface, they form a "doughnut" circular formation where cytotoxic activity is directly targeted at the target cell interface (Davis *et al.* 1999).

Peruzzi *et al.* reported interaction between KIR2DL1 and HLA-C which is characterized by the binding of KIR perpendicular to the α1 and α2 helices of HLA, as depicted in Figure 2.8, page 29 (Peruzzi *et al.* 1996). Receptor binding results in direct contact with peptide positions 7 and 8 at the C-terminal end of the HLA ligand (Peruzzi *et al.* 1996). Altered peptides that are bound in a particular groove can affect KIR recognition to the point where binding is inhibited by the particular ligand (Malnati *et al.* 1995). Lanier and co-workers proposed that peptides bound within the HLA groove function more in stabilizing the HLA molecules than the actual peptide configuration, while still having the capacity to affect KIR recognition (Lanier *et al.* 1998). KIR and TCR possess overlapping footprints and as such compete with each

other for the binding to the relevant HLA/peptide complex (Boyington *et al.* 2000). While KIR interacts with distal peptide positions, TCR binding sites overlap more centrally over peptide residues 4 to 6 (Boyington *et al.* 2000).

Boyington and co-workers proposed that crystal structures between KIR and HLA interact at a stoichoimetric ratio of 1:1 (Boyington *et al.* 2000, Fan *et al.* 2001).

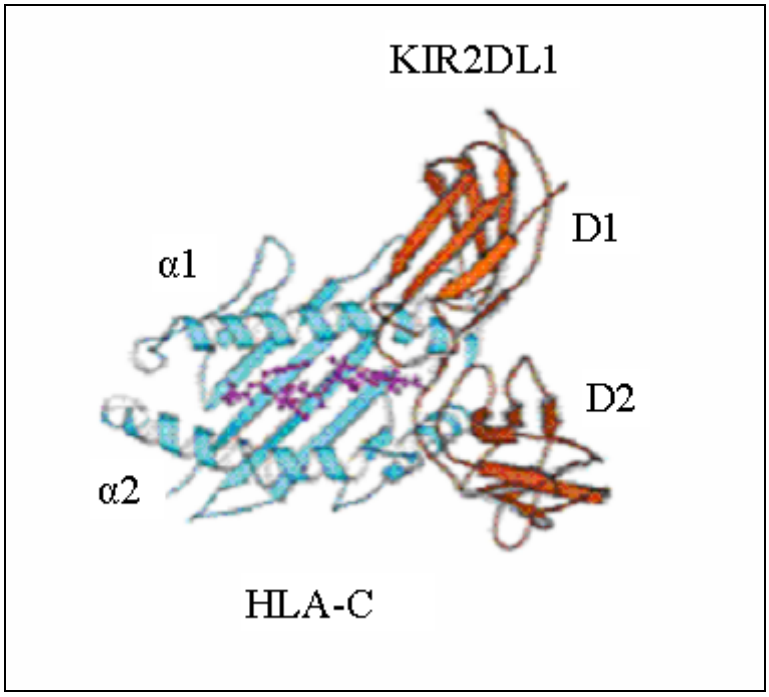

Figure 2.8 Overlapping footprint of both KIR/HLA (Figure was modified from Boyington *et al.* 2000)

Six loops from the KIR hinge region make contact with HLA. Of these, three loops (A'B, cc' and EF) are from the D1 domain, one from the hinge region and the final two (BC, FG) from the D2 domain (Figure 2.9, page 30) (Boyington *et al.* 2000). While the KIR interface between 2DL2 and HLA-Cw3 contains one basic (K44) and six acidic (E21, D72, E106, D135, D183 and E187) (indicated as red in Figure 2.9) residues, complementary HLA-Cw3 molecules have no acidic and six basic (R69, R75, R79, R145, K146 and R151) residues (indicated as blue in Figure 2.9).

Interactions result in the formation of four salt bridges (E21- R69; E106- R151; D135- R145 and D183- K146) (as shown by black arrows in Fgure 2.9). Although these molecules have poor complementary shapes, KIR/HLA interactions are strong as a result of the salt bridges (Boyington *et al.* 2000).

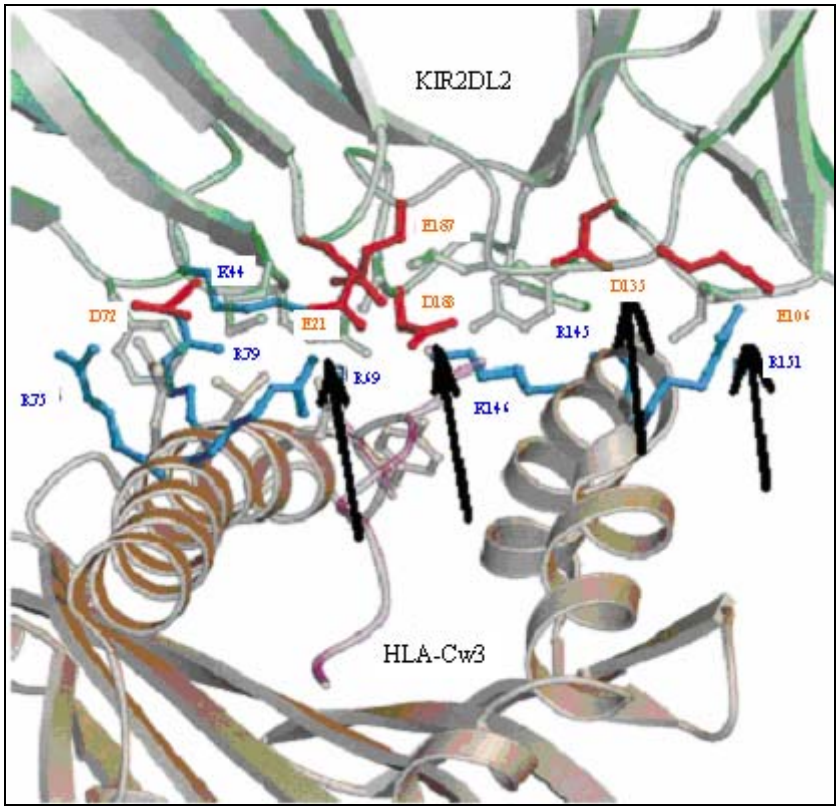

Figure 2.9 KIR/HLA interaction (Figure was modified and can be seen in Boyington *et al.* 2000) red = acidic ; blue = basic ; black arrows indicate salt briges.

Of the 13 HLA-Cw3 interface residues, amino acid 80 differs between HLA alleles, with variability between the C1 and C2 epitopes being Asn80 and Lys80 respectively (Cook *et al.* 2004). A hydrogen bond between Lys44 in 2DL2 and Asn80 in HLA-Cw3 are lost under circumstance of residue alterations resulting in ineffective binding and loss of specificity (Fan *et al.* 2001).

In the case of 2DL1 where Lys44 is replaced with a Met, the resulting effect is the receptor having specificity for C2 epitopes (Boyington *et al.* 2000). A further substitution where Lys80 replaces Asn80 also results in the loss of a hydrogen bond and ineffective binding between 2DL1 and the C2 epitopes (Winter *et al.* 1997).

The recognition of receptors is influenced by the affinity of KIR towards HLA. KIR receptors with greater HLA binding capacity receive preference over those with lower binding capacity, to the extent that receptors can be displaced by preferred receptors (Mandelboim *et al.* 1997). Mandelboim and co-workers proposed that peptides play alternative roles when recognised by different receptors, where binding of one receptor results in inhibition, binding with a different receptor result in activation (Mandelboim *et al.* 1997).

2.8 NK cell cytotoxicity

Cytotoxic effects of NK cells involve the use of two proteins, perforin and granzyme (Cosman *et al.* 1997). Both these proteins are present in lytic granules where they are stored until a target cell has been identified. Signalling responses alter intracellular calcium levels which result in the polarization and excretion of these lytic granules through exocytosis. Excretion of perforin and granzyme onto the KIR/HLA interface results in programmed cell death within the target cell. (Lieberman *et al.* 2003).

Perforin, once released onto the site of cell interface, forms a transmembrane pore which is assembled into the membrane of the target cell. This allows the free passage of salts, water and proteins into and out of the targeted cell disrupting its homeostatic barrier (Smith *et al.* 1987).

Granzyme, consists of three serine proteases that initiate apoptosis. Granzyme proteins enter through the pores created by perforin and activate an enzyme cascade resulting in DNA fragmentation. The first cascade enzyme is cysteine protease protein 32 (CPP-32), which is activated via cleavage. Activated CPP-32 then cleaves the inhibitory caspase-activated deoxyribonuclease (ICAD), which results in the dissociation of ICAD from caspase-activated deoxyribonuclease (CAD). CAD is translocated into the nucleus where it cleaves DNA into ~200 bp fragments, resulting in apoptosis (Smith *et al.* 1987).

Alternative ways in which immature NK cells (CD56 ^{dim}) can activate apoptosis include making use of TNF-related apoptosis-inducing ligand rather than by releasing perforin and granzyme (Loza *et al.* 2001). Tumour necrosis factor-α (TNF-α), which is present as a trimer can be expressed in both a soluble and a membrane bound form. Interaction between TNF-α and tumour necrosis factor receptor type-I (TNFR-I), which is also present as a trimer on the target cell, initiates apoptosis (Smith *et al.* 1987). The cytoplasmic domain of the TNFR-I is a "death domain" and interacts with pro-caspase-8 through the fas-associated death domain/tumour necrosis factor receptor type-I-associated death domain (FADD/TRADD) molecules resulting in initiation of the apoptosis cascade. After the activation of caspase-8, further activation of caspase-3 is accomplished which result in the degradation of I-CAD, allowing the activation of CAD. CAD is then transferred into the nucleus where DNA fragmentation occurs (Olcese *et al.* 1997).

Other receptors containing the "death domain" include the fas receptor (FasR) which interacts with fas ligand (FasL) located on NK cells, and as such initiates the enzyme cascade to activate CAD. Phagocytes such as macrophages rapidly ingest target cell fragments, limiting the inflammatory response (Smith *et al.* 1987).

Apart from possessing cytotoxic effects NK cells also play an alternative role in the innate immune system. They release cytokines and chemokines, which protects cells from further viral infection. IFN- γ that is released by NK cells up-regulates HLA Class I molecules as well as the degradation of ribonucleic acid (RNA) and DNA within the cytoplasm, thereby protecting these cells both internally and externally (Granucci *et al.* 2004).

2.9 Current research

Since its discovery in 1986, KIR has been recognised to play an important role in immunity (Harel-Bellan *et al.* 1986). The potential of KIR has only been realised recently, not only as having a vital role in the defence against viral infection but also in cancer and in particular, transplantation.

Recently, it has been shown that 3DS1 in combination with HLA-B with iso-leucine at position 80 resulted in delayed progression of AIDS after HIV infection (Martin *et al.* 2002b). Future studies may involve investigation of the KIR/HLA haplotypes of African sex workers who appear to have HIV resistance. Vaccines may be developed that stimulate the activating KIR repertoire to target HIV infected cells for destruction.

The study of KIR polymorphisms in relation to disease entities appears to be paralleling that of HLA; this is especially true of HIV. Organ transplantation has been affected by the knowledge of KIR and NK cells where bone marrow transplants (BMT) have shown higher engraftment rates when incompatible KIR donors are used with compatible HLA epitopes (Ruggeri *et al.* 1999).

Predictions towards future research are comparable to what has been observed in the field of HLA, where an explosion of interest in the polymorphism of disease in particular cancer and viral infections is imminent. Due to the potential role of KIR in transplantation, future discoveries should ultimately help in improving the quality of life for transplant patients as well as HIV sufferers.

2.10 KIR in transplantation

Possessing the ability to target cells for apoptosis, NK cells play a significant role in bone marrow transplantation. Genotyping for KIR allows for the opportunity to mismatch donor/recipient profiles, resulting in decreased marrow rejection, as compared to increased rejection in matched KIR transplants (Manilay *et al.* 1998).

There are many immunological variables in bone marrow transplantation and while limited studies have investigated the role of NK cells and KIR, increasing evidence indicates that NK cells plays a vital role in bone marrow engraftment (Manilay *et al.* 1998)

Bennette and co-workers reported that bone marrow transplantation from either parent to their offspring will result in recipient NK cells being inhibited by Class I antigens

from the other parent, therefore no NK cell response to their own bone marrow is anticipated (Bennette *et al.* 1995).

Van Der Meer and co-workers reported that most HLA-Cw mismatches induce only limited T cell responses (Van Der Meer *et al.* 2001). When bone marrow is also matched for other HLA antigens, KIR ligands induce NK cell lysis (Van Der Meer *et al.* 2001).

Grafts of solid organs such as kidney and heart have been noted for their infiltration by NK cells, usually before T cell infiltration (Baldwin *et al.* 2001). Transplant rejection through NK cells plays a greater role in bone marrow grafts than that of solid organs; although activated NK cells most likely influence the rejection process in some way (Baldwin *et al.* 2001).

Research within the fields of transplantation shows that allo-reactivity of NK cells towards leukaemic cells was of benefit to the patients as the Graft versus Leukaemic (GvL) effect protects against further recurrences of leukaemia (Ruggeri *et al.* 1999). This area of research may provide possible cures for cancer as well as other diseases.

2.11 KIR genotyping

Determination of KIR genes can be either locus or allele specific. Locus specific genotyping only detects presence or absence of a particular KIR gene within a given individual, thus producing a KIR haplotype profile. Allele specific genotyping makes use of more specific techniques in order to distinguish between various alleles at a locus level.

The polymerase chain reaction (PCR) provides a powerful approach in the genotyping of population cohorts. Being highly specific, PCR make use of both forward and reverse primers that bind to the KIR gene in question. Repetitive cycles of denaturation, annealing and extension allows for the exponential amplification of the gene in question. For a 35-cycle programme an estimated 34 billion copies are

produced if two strands are taken as initial templates. Refer to chapter 3 where cycle programs, reaction conditions and procedures are given for KIR genotyping.

The most appropriate method for KIR genotyping was SSP-PCR. This technique was first applied to KIR by Uhrberg *et al. (*1997). The normal PCR technique is still used, but SSP differs in primer design, where primers are human specific as well as being sequence specific for a particular KIR which ultimately only reacts to human KIR genes (Uhrberg *et al.* 1997). The selection of the SSP-PCR method was based on time, cost and infrastructure available.

An alternative genotyping method is sequence specific oligo-nuleotide probe-PCR (SSOP-PCR) (Uhrberg *et al.* 1997). This method makes use of an initial reaction that amplifies the entire region of KIR DNA, flanking all KIR genes. This is followed by a secondary addition of radioactive/fluorescent labelled "probes" targeting each KIR gene (Crum *et al.* 2000, Maxwell *et al.* 2003). SSOP-PCR detection methods include X-ray photography densometric readings when radioactive labels are used.

PCR and SSP-PCR make use of normal gel-electrophoresis. The latter method provides relative specificity and sensitivity while being cost effective when compared to other methods of product detection. Still more complex methods, such as sequencing or real time PCR provide increased specificity and sensitivity, however simplicity and cost are compromised.

The limitations of PCR include both sensitivity and specificity. Base pair alterations at an early cycle in the PCR are amplified to a substantial amount in later cycles. Further limitations include sensitivity where non-specific binding of primers result in variable fragment sizes when visualising products. Primer binding site mutations could also have the effect that false negatives can be observed.

2.12 Objectives

The aim was to establish a database of KIR frequencies within three Free State population groups (Caucasian, Black African and mixed ancestry). The project was aimed at investigating the diversity of KIR genes where haplotypes observed contributed to the construction of frequency tables.

The objective of the project was to develop and standardise an in-house KIR genotyping method with the purpose of saving costs, reagents and able adapted to equipment already present within the laboratory.

The standardisation of the in-house genotyping method also served as a pilot study to validate the correct allocation of KIR genes, as well as suitability of the method. Cohort samples were re-genotyped by Prof Derrek Middelton (National Blood Transfusion Service, Belfast, Northern Ireland) to validate the assignment of genotypes via the in-house method.

CHAPTER 3

Materials and Method

3.1 Introduction

SSP-PCR was the method of choice for the genotyping of the three ethnic cohorts where primer design, reaction conditions and cycling conditions can be noted in Tables 3.1 – 3.3. Initial experimentation was aimed at the standardising of KIR gene amplification, making use of primers described previously (Uhrberg *et al.* 1997, Norman *et al.* 2001, Hiby *et al.* 2004, Gómez-Lozano and Vilches *et al.* 2002). Development of this in-house genotyping method was performed in order to save costs while making use of equipment available in the laboratory. Random samples from all three cohorts were selected and verified using both the in-house method as well as the SSOP method, by Prof Derrek Middleton (National Blood Transfusion Service, Belfast Northern Ireland).

3.2 Study population

Genotyping was performed for three ethnic cohorts within central South Africa; black African, mixed ancestry and Caucasians. Fifty random samples where collected from each cohort with no preference towards age or sex.

3.3 Ethics of sample collection

Blood samples were obtained from the Paternity Testing Clinic at the Department of Haematology, University of the Free State. Ethics approval was obtained from the ethics committee (ETOVS 99/05) to use pre-collected samples. Samples were re-labelled from 1 to 50 for each cohort, in order to create an anonymous study population.

Table 3.1 Primers sets used for specific genes

Gene	Forward 5'-3'	Reverse 5'-3'	Amplicon Size (bp)
[a]2DS3	GAC ATG TAC CAT CTA TCC AC	GCA TCT GTA GGT TCC TCC T	130
[b] 2DS5	AGA GAG GGG ACG TTT AAC C	TCC AGA GGG TCA CTG GGC	172
[a] 3DL3	AAC ACG GAA CTT CCA AAT GCT GAG CG	GCA GGC AGT GGG GAC CTT AGA CA	243
[a] 3DS1	CAG CGC TGT GGT GCC TCG C	CTG TGA CCA TGA TCA CCA T	249
[a] 3DL1	AAG ACA CCC CCT ACA GAT ACC ATC T	GCA GGC AGT GGG GAC CTT AGA CA	277
[a] 2DP1	GCA AGA CAC CCC CAA CAG ATA CCA GA	GCA GGC AGT GGG GAC CTT AGA CA	278
[a] 3DP1	ATC CTG TGC GCT GCT GAG CTG AG	GCC TAT GAA AAC GGT GTT TCG GAA TAC	344
[a] 2DL3	CCT TCA TCG CTG GTG CTG	CAG GAG ACA ACT TTG GAT CA	812
[a] 2DL4	GTA TCG CCA GAC ACC TGC ATG CTG	GCA GGC AGT GGG GAC CTT AGA CA	1082
[a] 2DS2	CTT CTG CAC AGA GAG GGG AAG TA	CAC GCT CTC TCC TGC CAA	1761
[a] 2DL1	CTG TTA CTC ACT CCC CCT ATC AGG	AGG GCC CAG AGG AAA GTC A	1770
[a] 2DS1	TCT CCA TCA GTC GCA TGA A/G	AGG GCC CAG AGG AAA GTG/T	1838
[c] 2DL2	ACT TCC TTC TGC ACA GAG A A	GCC CTG CAG AGA ACC TAC A	1868
[a] 2DL5A	CTC CCG TGA TGT GGT CAA CAT GTA AA	GGG GTC ACA GGG CCC ATG AGG AT	1883
[a] 2DL5B	GTA CGT CAC CCT CCC ATG ATG TA	GGG GTC ACA GGG CCC ATG AGG AT	1893
[a] 2DS4	ATC CTG CAA TGT TGG TCG	CTG GAT AGA TGG TAC ATG TC	1902
[c] 3DL2	CGG TCC CTT GAT GCC TGT	GAC CAC ACG CAG GGC AG	1948

[a] Uhrberg *et al.* 1997 and Norman *et al.* 2001.

[b] Hiby *et al.* 2004.

[c] Gómez-Lozano and Vilches *et al.* 2002.

Table 3.2 PCR reaction conditions (final reaction volume = 10 µl + DNA volume negligible)

	2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1
10x Buffer (Takara) (µl)	1.2	1.2	-	1.0	1.2	1.2	1.1	1.0	-	1.2	-	-	-	1.2	-	-	-
10 x Buffer (Promega) (µl)	-	-	1.0	-	-	-	-	-	1.0	-	1.0	1.0	1.0	-	1.0	1.0	1.0
dNTPs (Takara) (µM)	250	250	-	200	250	250	250	250	-	250	-	-	-	250	-	-	-
dNTPs (Promega) (µM)	-	-	200	-	-	-	-	-	200	-	200	200	200	-	200	200	200
$MgCl_2$ (Promega) (mM)	-	1	3.5	-	-	1	1	-	3.5	-	3	4	2.5	1	3.5	3.5	3
Primer (pmol)	10	8	10	8	10	2	10	10	10	10	6	8	10	10	10	10	10
Internal Control Primer (ICP)	2DL4	2DL4	3DP1	-	2DL4	2DL4	2DL4	3DP1	3DP1	2DL4	3DL3	3DL3	3DP1	-	-	3DP1	-
Internal Control Primer (pmol)	2	8	7.5	-	2	2	10	10	10	5	6	10	10	-	-	5	-
DNA (ng)	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Taq Polymerase (Takara) (U)	2	2	-	2	2	2	2	2	-	2	-	-	-	2	-	-	-
Taq Polymerase (Promega) (U)	-	-	1	-	-	-	-	-	1	-	1	1	1	-	1	1	1

Table 3.3 PCR cycling conditions

		2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1
1x	1° Denaturation	95°C - 30s																
25x	2° Denaturation	95°C - 25s																
	Annealing	60.7°C 25s	59°C 25s	62.5°C 25s	60°C 25s	58°C 25s	63.8°C 25s	58.3°C 25s	63.5°C 25s	55°C 25s	57°C 25s	58.5°C 25s	59.5°C 25s	63°C 25s	62.2°C 25s	65°C 25s	61.8°C 25s	65°C 25s
	Extension	68°C 90s	68°C 100s	68°C 35s	68°C 90s	68°C 100s	68°C 105s	68°C 90s	68°C 90s	68°C 30s	68°C 105s	68°C 30s	68°C 30s	68°C 30s	68°C 100s	68°C 30s	68°C 30s	68°C 30s
5x	3° Denaturation	95°C - 20s																
	Annealing	55°C - 30s																
	Extension	68°C 100s	68°C 120s	68°C 60s	68°C 90s	68°C 110s	68°C 120s	68°C 120s	68°C 120s	68°C 30s	68°C 120s	68°C 30s	68°C 60s	68°C 60s	68°C 120s	68°C 60s	68°C 60s	68°C 60s
1x	Final Extension	68°C - 5 min																

s = sec
min = minutes

3.4 DNA isolation

Initial DNA samples were discarded and the second round were extracted using a Promega Wizard genomic DNA extraction kit (Promega Corporation, Madison, WI, USA) was used for the extraction of DNA from blood samples.

1. Add 900 µl of red cell lysis solution (RCLS) into a sterile 1.5 ml Eppendorf tube.
2. Add 300 µl of whole blood into RCLS, ensure that EDTA blood tubes are mixed.
3. Incubate for 10 minutes at room temperature with occasional mixing.
4. Centrifuge for 20 seconds at 8 000 g, decanting the supernatant upon completion.
5. Dislodge pellet by vortexing before adding 300 µl of nucleic lysis solution.
6. Mix with pipette until white cell pellet is completely dissolved.
7. Add 100 µl of protein precipitation solution and vortex until protein clumping occurs.
8. Centrifuge for 3 minutes at 8 000 g.
9. Decant supernatant into a new Eppendorf tube containing 300 µl iso-propanol.
10. Invert Eppendorf tube gently until strands become visible when held against the light.
11. Centrifuge samples for 1 minute at 8 000 g.
12. Decant supernatant allowing samples to dry for 1 hour at room temperature.
13. Add 100 µl DNA re-hydration solution to DNA samples and store at 4 degrees Celsius or at -4 degrees Celsius

3.5 DNA concentration measurement

DNA concentrations were determined prior to PCR reactions. Readings were taken on a spectrophotometer (Gene-Quant *Pro*, Biochron, Cambridge, England) at both A_{260} and A_{280} wavelengths and the ratio of these two readings were taken optical density. The reference reading was set with a dilution of 20 µl re-hydration solution (Promega Corporation) into 80 µl of distilled water. Readings for collected samples were taken

by first diluting 20 µl of DNA suspension into 80 µl of distilled water. Optical density (OD) readings were determined and multiplied by the dilution factor of 50 according to Equation 3.1. Only OD readings between 0.1 and 1 fall within the linear range of the optical density curve. Samples with readings larger than 1 were further diluted.

$$[\text{DNA}]\ \mu g/ml = \text{OD } A_{260} \times \text{dilution factor (df)} \times 50$$
$$(A_{260} = 1 \text{ is equivalent to } 50\ \mu g/ml \text{ of ds DNA})$$

Equation 3.1 Optical density calculations (Flavell *et al.* 1974)

DNA samples were diluted to a concentration of 30 ng/ul (Equation 3.2). Samples were stored at 4 ºC until PCR setup as per Section 3.6.

$$\text{Concentration}_{\text{Initial}} \times \text{Volume}_{\text{Initial}} = \text{Concentration}_{\text{Final}} \times \text{Volume}_{\text{Final}}$$

$$\text{Volume}_{\text{Initial}} = \frac{\text{Concentration}_{\text{Final}} \times \text{Volume}_{\text{Final}}}{\text{Concentration}_{\text{Initial}}}$$

$$\text{Volume}_{\text{Initial}} = \frac{30\ ng/\mu l \times 10\ \mu l}{OD \times df \times 50\ \mu g/ml}$$

Equation 3.2 DNA dilution calculations (Flavell *et al.* 1974)

3.6 PCR reaction conditions

Primers noted in Table 3.1 were synthesized by Integrated DNA Technology (IDT, Coralville, IA, USA) and diluted to a stock concentration of 100 mM with 1 x TE (10 mM of Tris-HCL [pH 8.3], 0.1 mM of EDTA) buffer. Working concentrations was made by diluting stock primer 1:10 with 1x TE. Tubes were labelled from one to fifty. Both a positive and a negative control sample were included. Reaction conditions were prepared on ice as noted in Table 3.2 and made up into a master mix, before dispensing 10 µl into each tube, with DNA being negligible towards the final reaction volume. Variable magnesium, buffer and primer concentrations were used to attain

optimal PCR product yield. DNA samples were thoroughly vortexed before being added.

In the first round of PCR reactions only the gene specific primer was used to determine the positive or negative samples for each gene. Internal control (IC) primers were added for the second round of PCR reactions in order to determine true negative results. For short length PCR products, KIR3DP1 and 3DL3 were used as internal control, while for long length products the framework primer 2DL4 was used. These primers were used as internal control as they amplify construction genes that are present within all individuals. Cycling was done on a Thermo Hybaid Px2 thermal cycler (Action Court, Ashford, Middlesex, TW15 1XB, UK) as noted in Table 3.3 with the inclusion of a "hot start" by allowing the PCR block to warm up before adding PCR reactions tubes.

3.7 Agarose gel electrophoresis of PCR products

PCR products were electrophoresed on 2 % agarose gels to determine the presence as well as the relative size of the amplified products. Agarose gels were prepared by microwaving 4 g (2 %) of agarose D1-LE (Whitehead Scientific) in 200 ml of 1x tris-borate-EDTA (TBE) (54 g of tris base, 27.5 g of boric acid, 20 ml of 0.5 M EDTA [pH 8.0], made up to 5 litres using ddH_2O, adjusted to pH 7.3) buffer until the solution became transparent with no visible clumps. Ethidium bromide (2 µl, 10 mg/ml) was added in order to visualize the DNA under UV light. Molecular-weight markers (Promega, G2101; MM, XII, VII) were added before electrophoresis at 8 V/cm for 15-25 minutes. After completion of the electophoresis, visualization of the DNA fragments was accomplished via UV transillumination.

3.8 Methodological errors associated with PCR results

DNA mutations may occur at primer binding sites, resulting in failure of primer binding. Other factors influencing PCR include DNA quality and fragmentation as too much vortexing can fragment DNA excessively. When quality deteriorates, where

possible, fresh DNA was isolated from stored blood samples before proceeding with genotyping.

Standardisation of reactions required different reagent concentrations to optimise PCR amplification conditions. Refer to Table 3.2 for specific PCR reaction conditions. Cycling conditions were optimal when non-specific products were avoided, while at the same time still annealing to KIR genes. Extension time for the cycling conditions was also adjusted to match the product size.

3.9 Comparative PCR methods

Validation of in-house genotyping required comparative identification, which made use of genotyped results from Prof Derrek Middleton (National Blood Transfusion Service, Belfast, Northern Ireland) indicating the correct gene assignment. A total of 30 samples were randomly selected for this purpose without the knowledge of each sample haplotype, 10 samples from each ethnic cohort and are indicated with bold text in Tables 4.1 to 4.3. Results from both methods were done as a blind sample study and there were no discrepancies observed.

3.10 Statistical analysis

Statistical analyses were performed to study the two-locus gene association within each cohort, as well as testing for inheritance probability. Frequency graphs (appendix A) were constructed to identify the occurrence of each particular gene and to create a database for each of the cohorts. KIR locus frequency (KLF) calculations determining the frequencies for the putative loci were calculated using the formula noted in Equation 3.3, where (f) is the observed frequency.

$$KLF = 1\sqrt{(1-f)}$$

Equation 3.3 Calculation for KIR locus frequency (De Kock *et al.* 1997)

The particular KIR genotype of each individual was shown by the presence (■) or absence (□) of each KIR gene and can be noted in Tables 4.1 to 4.3. The relevant data was tabulated in a spreadsheet for further calculation and can be noted in appendix A.

The Chi^2 test was used to compare observed and expected frequencies (Spiegel 1968). Observed frequencies were obtained by counting the number of times two genes occur in the same haplotype. Expected values were obtained by multiplying the specific allele frequencies with one another. Chi^2 calculations (h) were performed according to Equation 3.4 where the differences between observed and expected values are squared before being divided by the expected value.

$$Chi^2 = \frac{(Obs - Exp)^2}{Exp}$$

Equations 3.4 Calculation for Chi^2 (Spiegel 1968)

Yates contingency tables were used to compare the observed and expected results and the level of significance (Yates 1934). The probabilities (*p*) determining the level of significance were calculated using one degree of freedom as indicated in Tables 4.6 to 4.8.

Further comparative observations revealed relative association of two-locus inheritance with respect to each other. This was measured using linkage disequilibrium as calculated by Martin and co-workers (Martin *et al.* 2000). The delta value (Δ), measures the strength of association (Equation 3.5). Linkage disequilibrium values (Δ_{AB}) were calculated by subtracting the expected ($P_A * P_B$) frequency from the observed ($P_{(AB)}$) frequency.

$$\Delta_{(AB)} = (P_{(AB)}) - (P_{(A)} * P_{(B)})$$

$$\Delta_{(max)} = \text{if } \Delta < 0 = \text{Min } (P_A * P_B, (1-P_A)*(1-P_B))$$

$$= \text{if } \Delta > 0 = \text{Max } ((1-P_A)*P_B, P_A*(1-P_B))$$

Equation 3.5 Linkage disequilibrium calculation (Martin *et al.* 2000)

The absolute delta did not allow for different loci pairs to be compared and as such relative (r) linkage disequilibrium was previously reported (Baur and Danilovs 1980, Lewontin 1964). A normalised Δ' value is calculated by dividing Δ with the theoretical Δ_{max} as stated in Equation 3.6.

$$\Delta' = \Delta/\Delta_{max}$$

Equation 3.6 Relative linkage disequilibrium calculation (Baur and Danilovs 1980, Lewontin 1964)

Normalised Δ' linkage disequilibrium indicates the degree of association depending on the value obtained, where 1 is the threshold resulting in loci being in complete linkage disequilibrium.

CHAPTER 4

Results

4.1 KIR haplotype results

Table 4.1 to 4.3 represent all KIR haplotypes detected for a total of 150 samples, where each cohort was limited to 50 samples which was sufficient as to stabilize frequency variation. While smaller populations would not have given accurate frequency results, larger sample populations would have required increased time and cost.

As noted in Table 4.1 to 4.3, all framework genes are present throughout the three ethnic cohorts. These findings were expected for the black African and mixed ancestry cohorts and are reported for Caucasians (Uhrberg *et al.* 1997, Crum *et al.* 2000, Witt *et al.* 1999, Norman *et al.* 2001, Toneva *et al.* 2001, Niokou *et al.* 2003). Other variable genes are present in a fractional proportion within the haplotype pool. The number of KIR genes present on a single haplotype varied between 6 and 15 as were found within all three ethnic cohorts with an average value of 11 genes per haplotype.

A summary of all haplotypes encountered during the genotyping of all three cohorts are presented in Table 4.4 where the number of loci, haplotypic profile and frequency % are listed. As noted 64 new KIR haplotypes have been identified with 42 of these from the mixed ancestry cohort and the other 22 from the black African and Caucasian populations.

Table 4.1 KIR haplotype results for the Caucasian population

	2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1
1	■		■	■			■			■		■	■	■	■	■	■
2	■		■	■			■			■		■	■	■	■	■	■
3	■		■	■			■			■		■	■	■	■		■
4	■	■	■	■			■	■		■		■	■	■	■		■
5	■	■	■	■	■			■		■	■	■	■	■	■	■	■
6	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■
7	■	■	■	■	■		■	■	■		■	■		■	■	■	■
8	■		■	■						■		■	■	■	■		■
9	■		■	■	■		■			■	■	■	■	■	■	■	■
10	■	■		■	■		■	■	■	■		■	■	■	■	■	■
11	■		■	■	■		■			■		■	■	■	■	■	■
12	■		■	■						■		■	■	■	■	■	■
13	■	■	■	■		■	■	■		■	■	■	■	■	■	■	■
14	■	■	■	■			■	■		■		■	■	■	■	■	■
15	■	■		■			■	■		■		■	■	■	■	■	■
16	■		■	■						■		■	■	■	■		■
17	■		■	■			■			■		■	■	■	■	■	■
18	■		■	■			■			■		■	■	■	■	■	■
19	■	■	■	■				■	■	■		■	■	■	■	■	■
20	■		■	■						■		■	■	■	■	■	■
21	■		■	■						■		■	■	■	■	■	■
22	■	■	■	■		■	■	■		■	■	■	■	■	■	■	■
23	■		■	■			■					■	■	■	■	■	■
24	■		■	■			■			■		■	■	■	■	■	■
25	■		■	■						■		■	■	■	■	■	■
26	■		■	■		■	■			■	■	■	■	■	■	■	■
27	■		■	■		■	■			■	■	■	■	■	■	■	■
28	■		■	■		■				■	■	■	■	■	■	■	■
29	■		■	■						■		■	■	■	■	■	■
30	■		■	■		■	■			■	■	■	■	■	■	■	■
31	■	■	■	■		■		■		■	■	■	■	■	■	■	■
32	■		■	■						■		■	■	■	■	■	■
33				■	■		■		■	■		■	■	■	■	■	■
34			■	■						■		■	■	■	■	■	■
35	■		■	■			■			■		■	■	■	■	■	■
36	■		■	■						■			■	■	■	■	■
37	■		■	■						■		■	■	■	■		■
38	■		■	■						■		■	■	■	■		■
39	■		■	■						■		■	■	■	■		■
40	■	■	■	■	■			■		■	■	■	■	■	■	■	■
41	■	■	■	■	■		■	■		■	■	■	■	■	■	■	■
42	■		■	■						■		■	■	■	■		■
43	■			■			■			■		■	■	■	■		■
44	■	■	■	■			■	■				■	■	■	■		■
45	■		■	■			■			■		■	■	■	■	■	■
46	■		■	■						■		■	■	■	■	■	■
47	■		■	■						■		■	■	■	■		■
48	■		■	■						■		■	■	■	■		■
49	■	■	■	■				■		■		■	■	■	■		■
50			■	■								■		■	■		■

Table 4.2 KIR haplotype results for the Black African population

	2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1
1	■		■	■	■					■	■	■	■	■	■		■
2	■			■	■					■	■	■	■	■	■		■
3	■	■		■	■			■		■	■	■	■	■	■		■
4	■		■	■			■			■		■	■	■	■		■
5	■		■	■		■				■	■	■	■	■	■		■
6	■		■	■						■		■	■	■	■		■
7	■	■	■	■		■		■	■	■	■	■	■	■	■	■	■
8	■			■		■			■	■	■	■	■	■	■	■	■
9		■	■	■		■		■		■	■	■	■	■	■		■
10			■	■						■		■	■	■	■	■	■
11	■		■	■						■		■	■	■	■		■
12	■		■	■						■		■	■	■	■		■
13	■			■		■	■			■	■	■	■	■	■		■
14	■			■	■		■			■		■	■	■	■		■
15	■	■		■	■		■	■		■	■	■	■	■	■		■
16	■		■	■						■		■	■	■	■	■	■
17	■	■	■	■				■		■		■	■	■	■	■	■
18	■			■						■		■	■	■	■		■
19			■	■		■				■	■	■		■	■	■	■
20			■	■	■					■	■	■		■	■		■
21	■	■		■	■			■	■	■	■	■	■	■	■		■
22			■	■	■		■			■	■	■		■	■		■
23			■	■		■	■			■	■	■	■	■	■		■
24	■		■	■		■				■	■	■	■	■	■		■
25	■		■	■	■		■			■	■	■	■	■	■		■
26	■	■	■	■		■	■	■		■	■	■	■	■	■		■
27	■	■	■	■	■			■		■	■	■	■	■	■	■	■
28			■	■						■		■	■	■	■	■	■
29			■	■		■				■	■	■	■	■	■	■	■
30	■	■	■	■		■	■	■		■	■	■	■	■	■	■	■
31	■		■	■						■		■	■	■	■		■
32	■	■	■	■				■		■		■	■	■	■		■
33	■			■			■			■		■	■	■	■		■
34			■	■						■		■	■	■	■		■
35	■		■	■			■			■		■	■	■	■		■
36	■			■						■		■	■	■	■		■
37	■			■	■		■					■	■	■	■		■
38	■		■	■						■		■	■	■	■	■	■
39	■	■	■	■			■	■		■		■	■	■	■	■	■
40	■	■	■	■				■		■		■	■	■	■		■
41	■	■	■	■			■	■		■		■	■	■	■	■	■
42	■			■			■		■	■		■	■	■	■	■	■
43	■	■	■	■		■		■	■	■	■	■	■	■	■	■	■
44	■		■	■		■				■	■	■	■	■	■		■
45	■	■	■	■				■		■		■	■	■	■		■
46	■	■	■	■				■		■		■	■	■	■		■
47	■		■	■						■		■	■	■	■		■
48	■		■	■						■		■	■	■	■		■
49	■	■	■	■	■			■		■		■	■	■	■	■	■
50	■			■						■			■	■	■		■

Table 4.3 KIR haplotype results for the mixed ancestry population

	2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1
1			■	■								■	■	■	■	■	■
2			■	■			■			■			■	■	■	■	■
3			■	■		■					■	■	■	■	■	■	■
4			■	■			■		■	■		■	■	■	■		■
5				■						■		■	■	■	■	■	■
6	■			■				■		■		■	■	■	■	■	■
7				■			■			■		■	■	■	■	■	■
8			■	■		■				■	■	■	■	■	■		■
9		■		■						■		■	■	■	■	■	■
10	■	■	■	■		■	■			■	■	■	■	■	■	■	■
11		■	■	■	■	■				■	■	■	■	■	■		■
12			■	■				■		■		■	■	■	■		■
13	■	■	■	■	■					■		■	■	■	■		■
14	■	■		■	■			■		■		■	■	■	■		■
15			■	■				■		■		■		■	■	■	■
16				■				■		■		■	■	■	■	■	■
17				■						■		■	■	■	■	■	■
18		■	■	■						■		■	■	■	■	■	■
19			■	■						■		■	■	■	■	■	■
20	■	■		■	■			■		■		■	■	■	■	■	■
21	■	■		■	■					■		■	■	■	■	■	■
22			■	■	■	■				■	■	■	■	■	■		■
23		■	■	■		■				■	■	■	■	■	■	■	■
24				■	■				■	■		■		■	■		■
25				■						■		■		■	■		■
26				■	■	■				■	■	■	■	■	■		■
27	■			■						■		■	■	■	■		■
28				■						■		■	■	■	■		■
29			■	■					■	■		■	■	■	■	■	■
30				■	■	■			■	■	■	■	■	■	■	■	■
31	■	■		■	■					■	■	■	■	■	■		■
32				■						■		■	■	■	■		■
33		■		■		■			■	■	■	■	■	■	■	■	■
34	■	■		■	■				■	■	■	■	■	■	■	■	■
35				■		■				■	■		■	■	■	■	■
36				■		■			■	■	■	■	■	■	■		■
37	■	■	■	■						■		■	■	■	■		■
38	■	■	■	■						■		■	■	■	■		■
39			■	■						■		■	■	■	■		■
40		■	■	■						■			■	■	■		■
41				■	■				■	■			■	■	■		■
42	■	■		■		■				■	■	■	■	■	■		■
43			■	■	■	■			■	■	■	■	■	■	■	■	■
44			■	■		■			■	■	■		■	■	■	■	■
45			■	■						■			■	■	■		■
46			■	■		■				■	■	■	■	■	■	■	■
47	■	■	■	■					■	■	■	■	■	■	■	■	■
48				■					■	■		■	■	■	■		■
49	■			■		■				■	■	■	■	■	■		■
50				■	■	■				■	■	■	■	■	■		■

Table 4.4 Summary of all KIR haplotypes observed

	Haplotype	Frequency (n)		No of Loci	2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1
1	AB1	1	S-1	13																	
2	AB2	1	S-1	14																	
3	AB4	3	S-3	12																	
4	AB5	1	B-1	12																	
5	AB6	3	S-1, B-2	14																	
6	AB7	1	B-1	16																	
7	AB8	1	B-1	14																	
8	AB9	3	S-1, B-2	15																	
9	AB10	1	K-1	11																	
10	AB11	2	K-2	10																	
11	AB12	1	B-1	14																	
12	AB13	1	K-1	13																	
13	AB14	1	K-1	14																	
14	AB15	3	S-2, B-1	13																	
15	AB16	1	B-1	13																	
16	AB17	1	B-1	11																	
17	AB18	3	S-2, B-1	11																	
18	AB19	1	B-1	15																	
19	AB20	2	S-2	15																	
20	AA1	6	B-6	11																	
21	AA2	4	S-3, B-1	10																	
22	AA3	15	S-7, B-8	9																	
23	AA4	1	B-1	13																	
24	AA5	2	B-2	12																	
25	AA6	8	S-2, B-6	10																	
26	AA8	1	B-1	10																	
27	AA9	4	B-4	13																	
28	AA10	1	B-1	12																	
29	AA11	1	B-1	9																	
30	AA12	1	S-1	11																	
31	AA13	3	S-3	11																	
32	AA14	1	S-1	12																	
33	C1	1	S-1	10																	
34	C2	2	S-1, B-1	9																	
35	C3	1	S-1	10																	
36	C4	1	S-1	11																	
37	C5	1	S-1	7																	
38	C6	1	K-1	10																	
39	C7	2	K-1, S-1	8																	
40	C8	1	K-1	10																	
41	C9	1	S-1	12																	
42	C10	1	S-1	11																	
43	C11	1	B-1	12																	
44	C12	1	S-1	12																	
45	C13	1	K-1	11																	
46	C14	1	S-1	13																	
47	C15	1	K-1	11																	
48	C16	1	K-1	13																	
49	CC17	1	K-1	11																	
50	CC18	1	K-1	12																	
51	CC19	1	K-1	11																	
52	CC20	1	S-2	13																	
53	CC21	1	B-1	14																	
54	CC23	1	K-1	9																	
55	CC24	1	K-1	8																	
56	CC25	1	K-1	6																	
57	CC27	1	K-1	8																	
58	CC28	1	B-1	11																	
59	CC29	2	K-2	7																	
60	CC30	1	K-1	9																	
61	CC31	1	K-1	12																	
62	CC32	1	K-1	10																	
63	CC33	1	K-1	9																	
64	CC35	2	K-2	8																	
65	CC36	1	K-1	8																	
66	CC37	2	K-2	10																	
67	CC38	1	K-1	8																	
68	CC39	1	K-1	9																	
69	CC40	1	K-1	10																	
70	CC41	1	K-1	10																	
71	CC42	1	S-1	10																	
72	CC43	1	K-1	9																	
73	CC44	1	K-1	9																	
74	CC45	4	K-1, S-2, B-1	9																	
75	CC46	1	K-1	11																	
76	CC47	1	K-1	10																	
77	CC48	2	K-1, S-1	8																	
78	CC49	1	K-1	13																	
79	CC50	1	K-1	11																	
80	CC51	1	K-1	7																	
81	CC52	1	K-1	10																	
82	CC53	2	K-1, S-1	11																	
83	CC54	1	K-1	8																	
84	BB1	1	K-1	12																	
85	CC56	1	K-1	12																	
86	BB2	1	K-1	9																	
87	CC58	1	K-1	12																	
88	CC59	1	K-1	10																	
89	CC60	1	S-1	12																	
90	CC61	1	B-1	6																	
91	CC62	1	S-1	11																	
92	CC63	1	S-1	10																	
93	CC64	1	S-1	9																	
		TOTAL;150		AVE; 10.8																	

S = African black; K = Mixed ancestry; B = Caucasians

4.2 PCR products

Photographed results are represented in figures 4.1 (a-q), where examples of PCR products for each typed gene can be noted. DNA ladders indicate relative fragment size. Each lane is represented by a single sample. Samples with no PCR product were counted as a negative only when the internal control fragments are present, thus indicating that the PCR reaction was successful and no false negatives were reported.

Internal control fragment sizes are illustrated on the photographed samples to show the correct product size. Internal control fragments were observed to be larger than or smaller than the gene fragment. This depends on the size of the gene and the internal control fragment. Product fragments are visualized under UV light and Polaroid photographs taken. Each cohort was analysed separately for each KIR gene.

Both negative and positive control samples were included within each cohort batch, as additional PCR controls. Negative control samples were prepared as described in Section 3.6, except that the DNA was substituted by double distilled water. No fragments were observed in any negative control lanes (not represented in illustrated pictorials). Positive control samples were obtained from Prof Derrek Middleton (National Blood Transfusion Service, Belfast Northern Ireland) and were processed the same as normal samples (Section 3.6). Complete batch results were discarded when either positive or negative controls failed. These indicators provided information for both false positive scoring and inadequate processing.

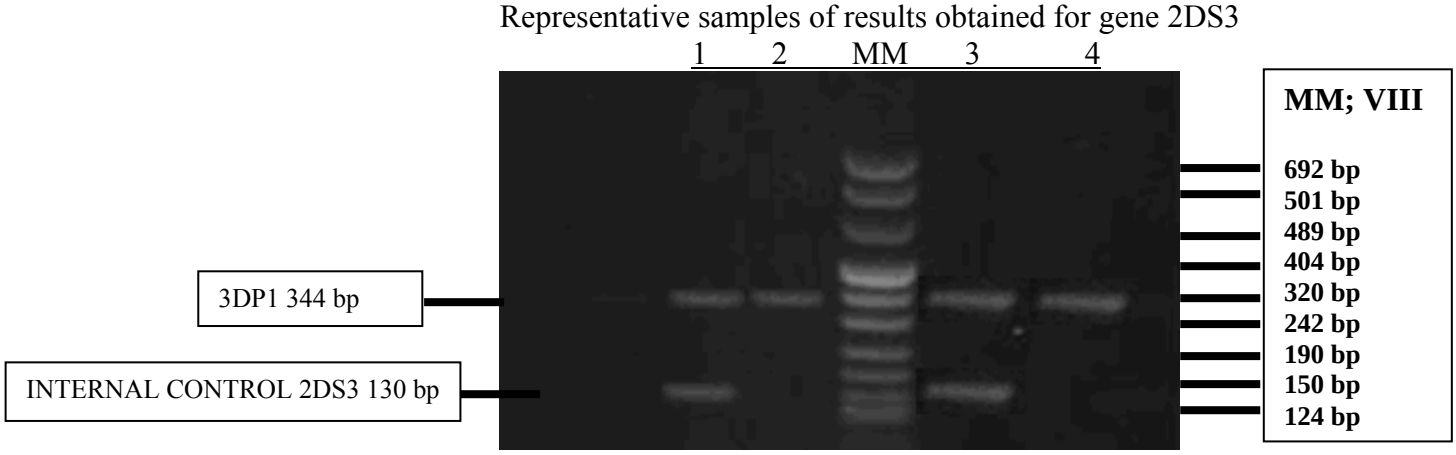

Figure 4.1 (a) Photograph of 2DS3 gene PCR product
2 % agarose gel, 8 V/cm for 30 min, MM = Molecular Marker = VIII
G2101, Lane 1 = POS CONTROL, NEG CONTROL not indicated

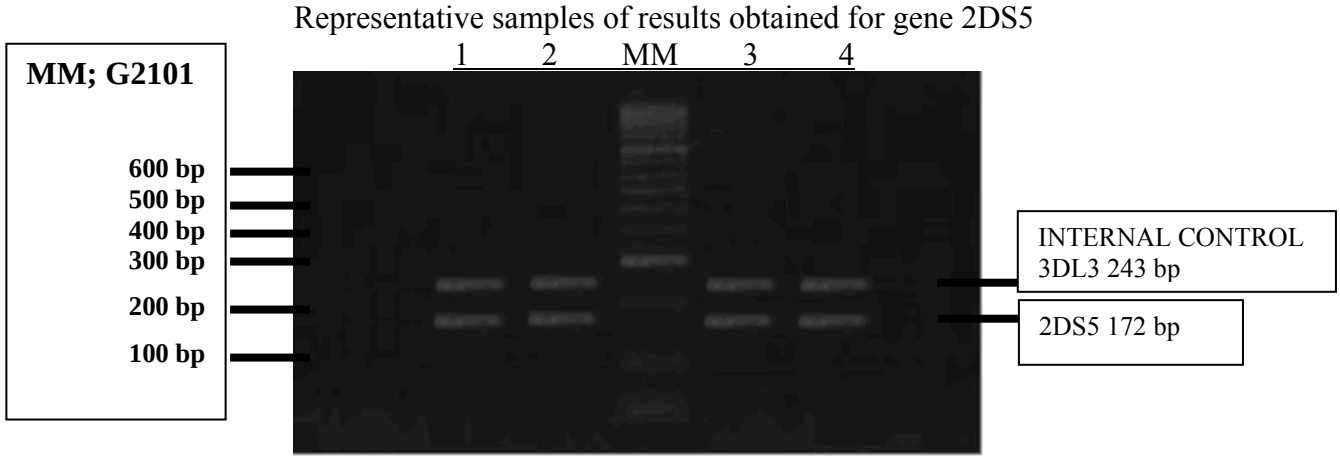

Figure 4.1 (b) Photograph of 2DS5 gene PCR product
2 % agarose gel, 8 V/cm for 30 min, MM = Molecular Marker =
G2101, Lane 1 = POS CONTROL, NEG CONTROL not indicated

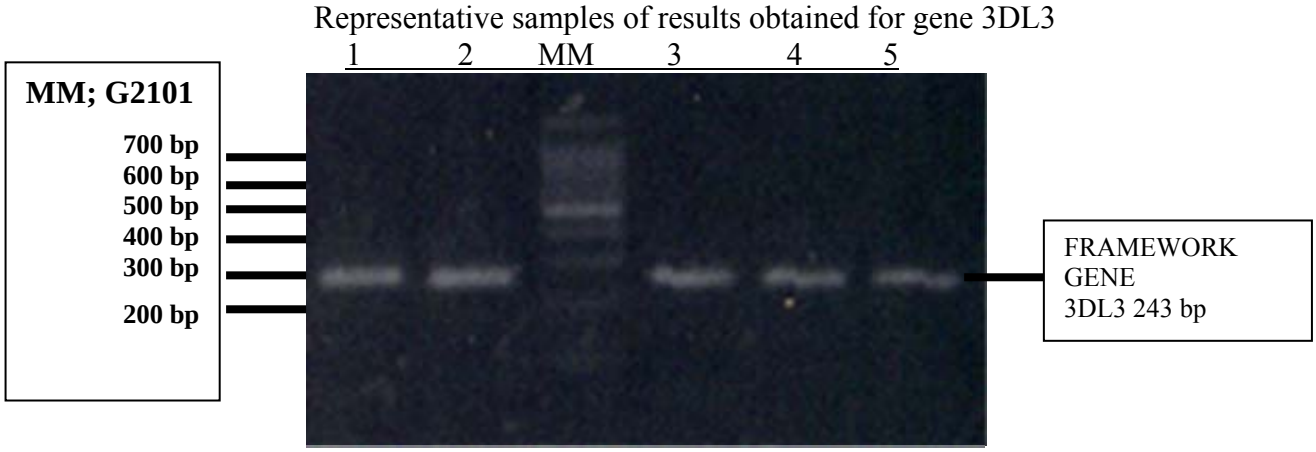

Figure 4.1 (c) Photograph of 3DL3 gene PCR product
2 % agarose gel, 8 V/cm for 30 min, MM = Molecular Marker = G2101, Lane 1 = POS CONTROL, NEG CONTROL not indicated

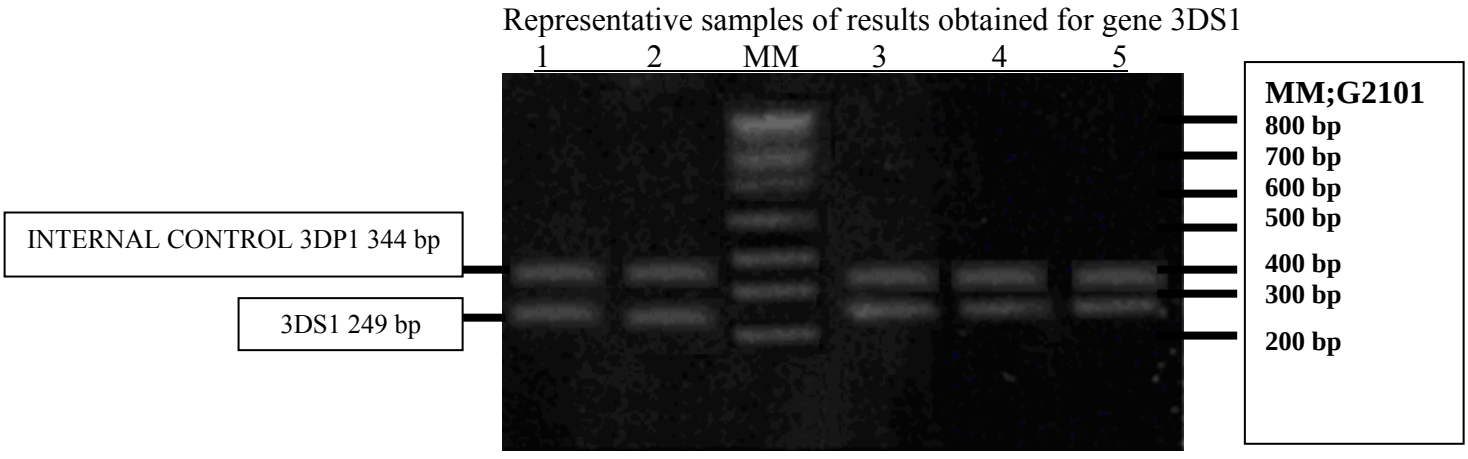

Figure 4.1 (d) Photograph of 3DS1 gene PCR product
2 % agarose gel, 8 V/cm for 30 min, MM = Molecular Marker = G2101, Lane 1 = POS CONTROL, NEG CONTROL not indicated

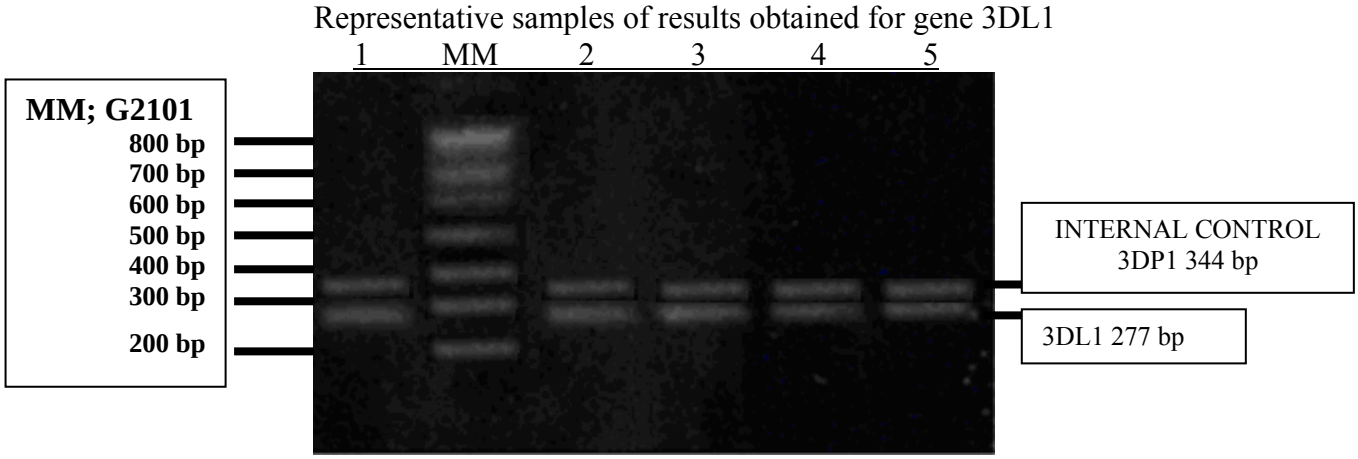

Figure 4.1 (e) Photograph of 3DL1 gene PCR product
2 % agarose gel, 8 V/cm for 30 min, MM = Molecular Marker = G2101, Lane 1 = POS CONTROL, NEG CONTROL not indicated

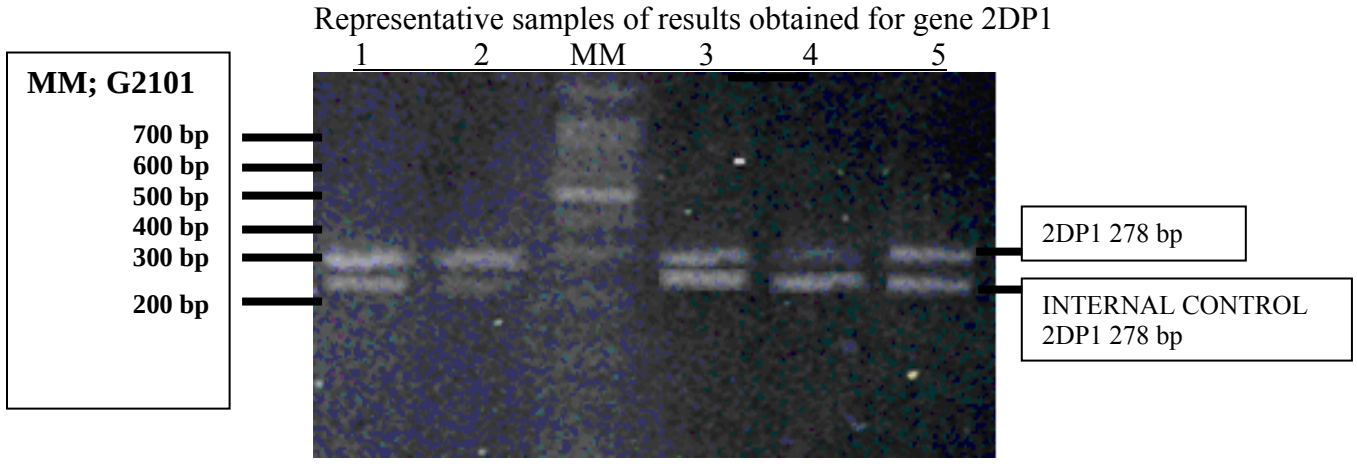

Figure 4.1 (f) Photograph of 2DP1 gene PCR product
2 % agarose gel, 8 V/cm for 30 min, MM = Molecular Marker = G2101, Lane 1 = POS CONTROL, NEG CONTROL not indicated

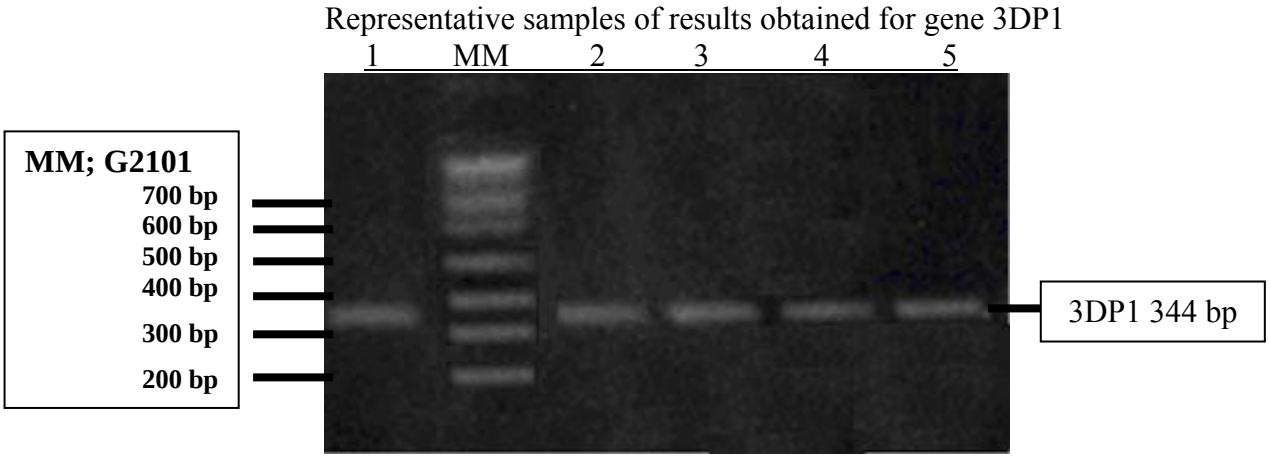

Figure 4.1 (g) Photograph of 3DP1 gene PCR product
2 % agarose gel, 8 V/cm for 30 min, MM = Molecular Marker =G2101
Lane 1 = POS CONTROL, NEG CONTROL not indicated

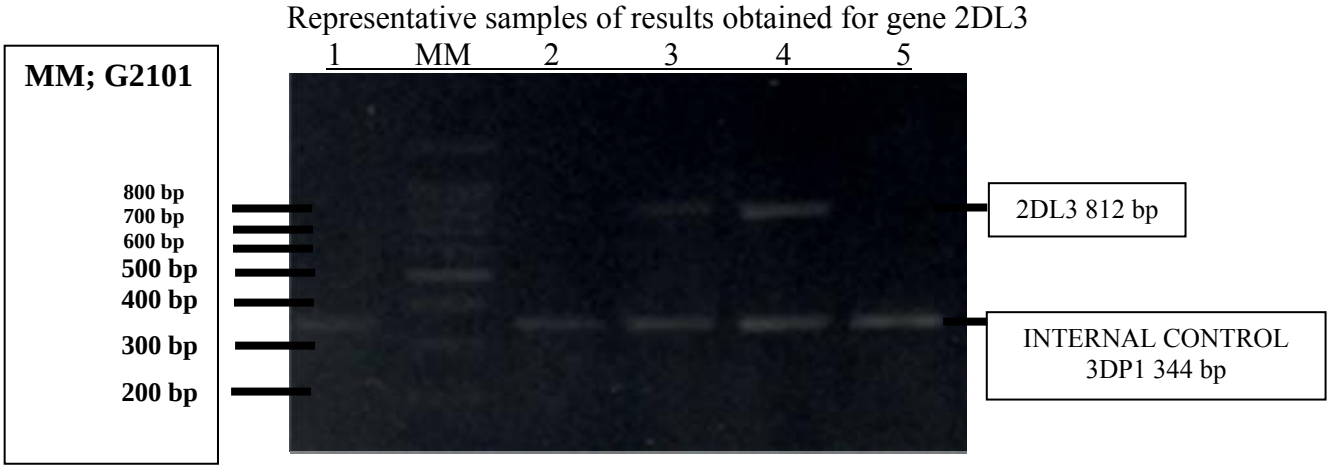

Figure 4.1 (h) Photograph of 2DL3 gene PCR product
2 % agarose gel, 8 V/cm for 1 hour, MM = Molecular Marker = G2101
Lane 1 = POS CONTROL, NEG CONTROL not indicated

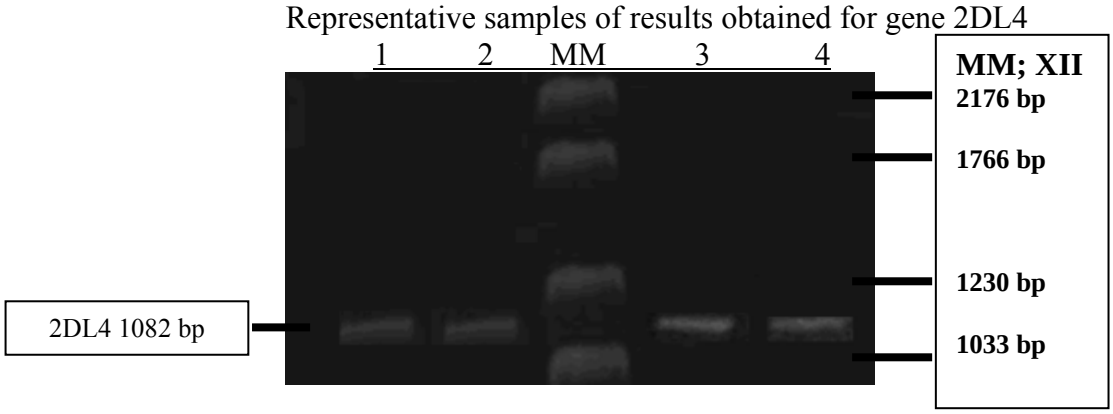

Figure 4.1 (i) Photograph of 2DL4 gene PCR product
2 % agarose gel, 8 V/cm for 2 hour, MM = Molecular Marker = XII
Lane 1 = POS CONTROL, NEG CONTROL not indicated

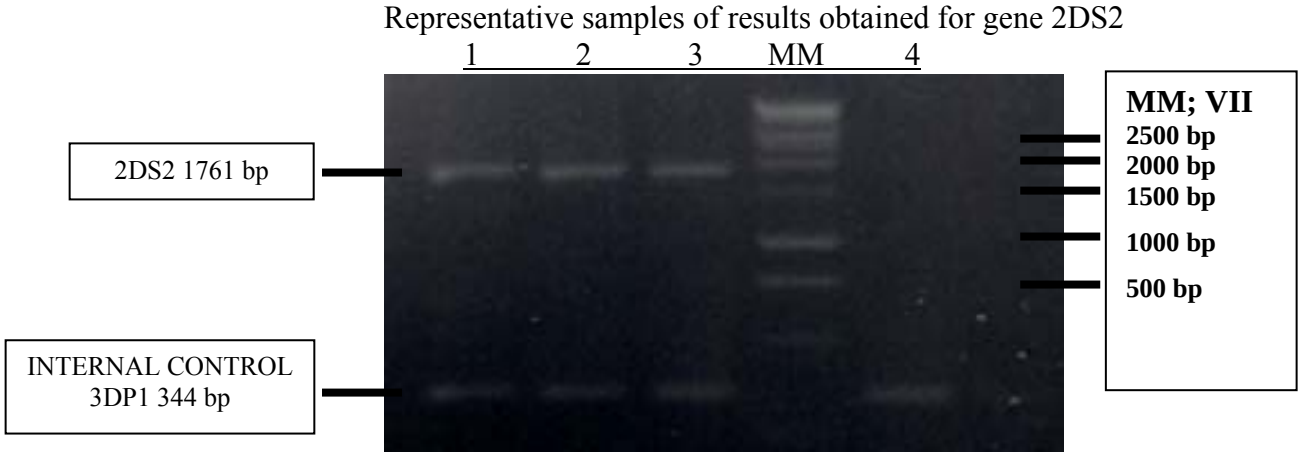

Figure 4.1 (j) Photograph of 2DS2 gene PCR product
1.5 % agarose gel, 8 V/cm for 1 hour, MM = Molecular Marker = VII
Lane 1 = POS CONTROL, NEG CONTROL not indicated

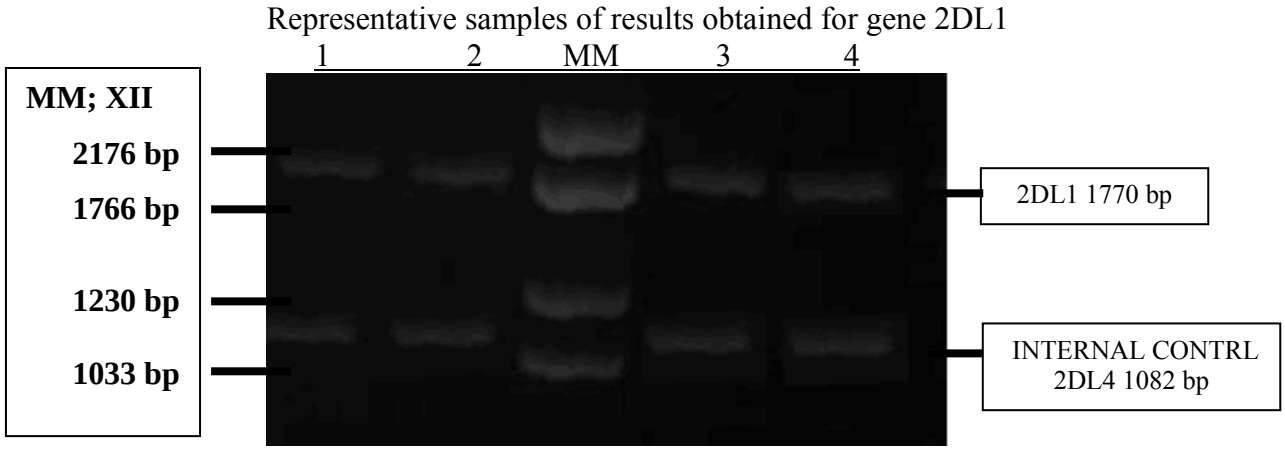

Figure 4.1 (k) Photograph of 2DL1 gene PCR product
1.5 % agarose gel, 8 V/cm for 2 hour, MM = Molecular Marker = XII
Lane 1 = POS CONTROL, NEG CONTROL not indicated

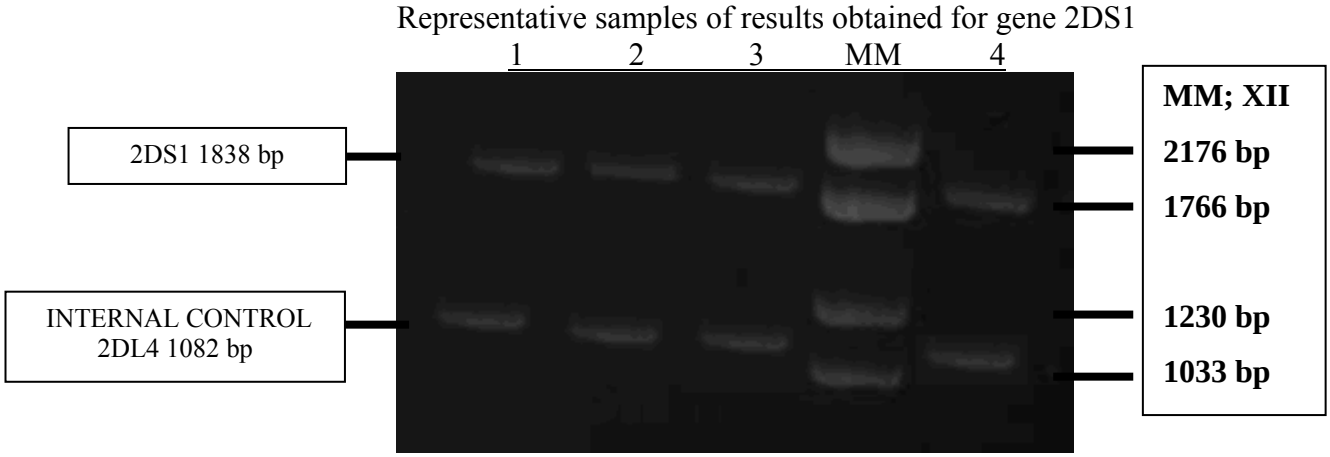

Figure 4.1 (l) Photograph of 2DS1 gene PCR product
1.5 % agarose gel, 8 V/cm for 2 hour, MM = Molecular Marker = XII
Lane 1 = POS CONTROL, NEG CONTROL not indicated

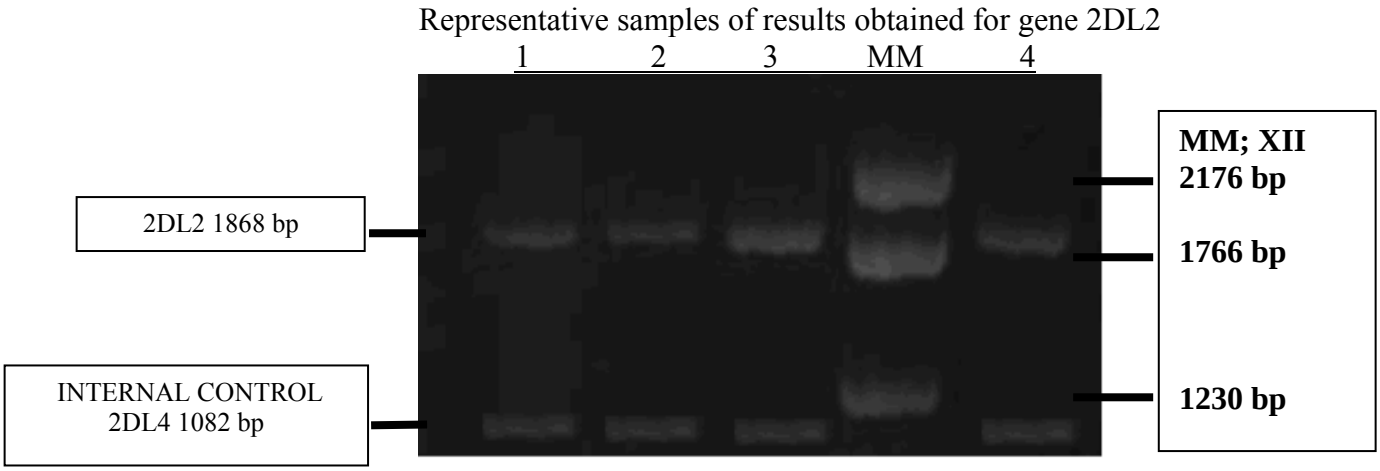

Figure 4.1 (m) Photograph of 2DL2 gene PCR product
1.5 % agarose gel, 8 V/cm for 2 hour, MM = Molecular Marker =XII
Lane 1 = POS CONTROL, NEG CONTROL not indicated

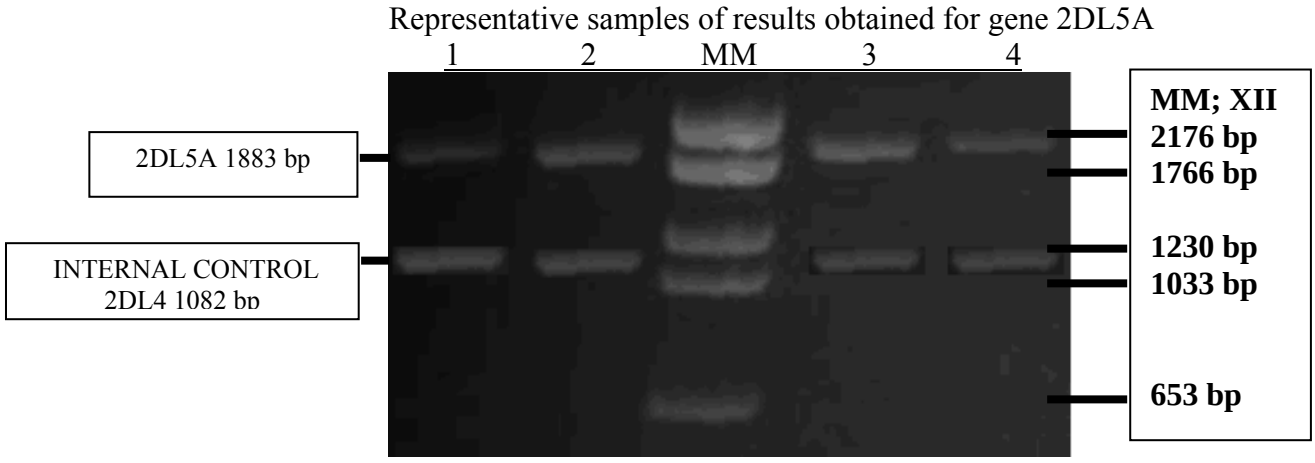

Figure 4.1 (n) Photograph of 2DL5A gene PCR product
1.5 % agarose gel, 8 V/cm for 2 hour, MM = Molecular Marker = XII
Lane 1 = POS CONTROL, NEG CONTROL not indicated

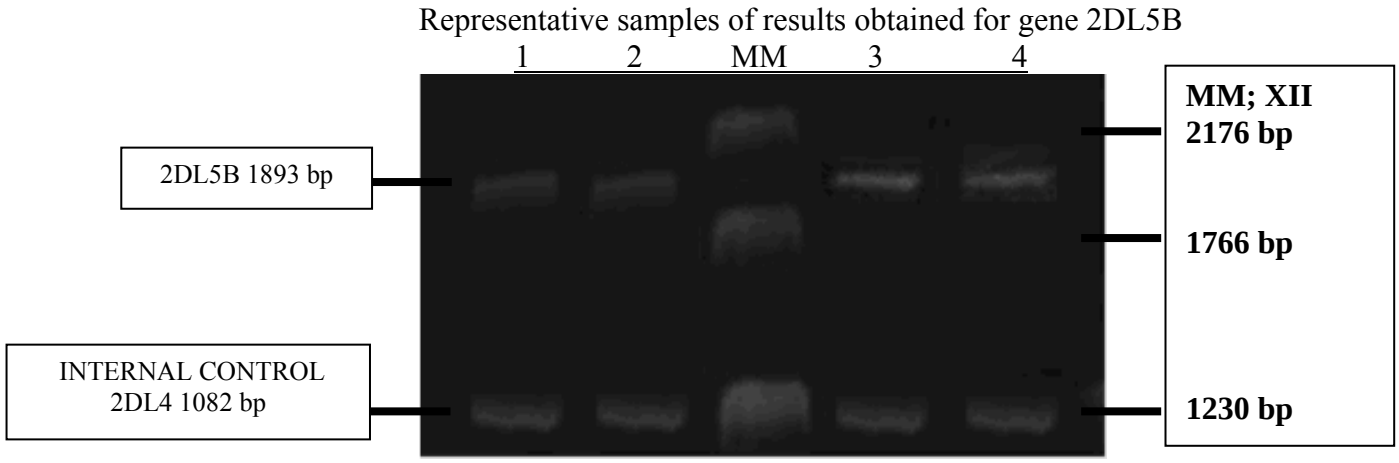

Figure 4.1 (o) Photograph of 2DL5B gene PCR product
1.5 % agarose gel, 8 V/cm for 2 hour, MM = Molecular Marker = XII
Lane 1 = POS CONTROL, NEG CONTROL not indicated

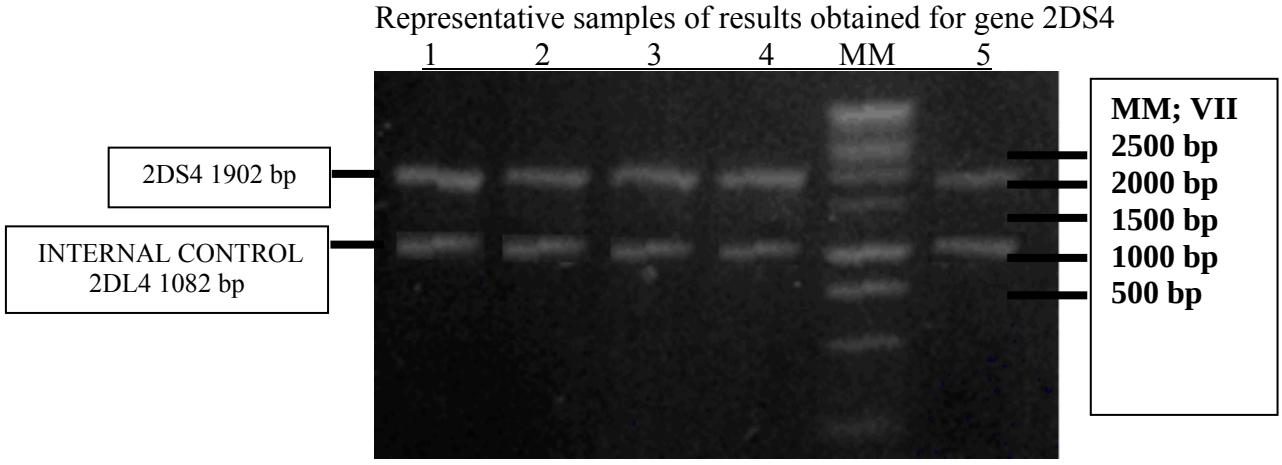

Figure 4.1 (p) Photograph of 2DS4 gene PCR product
1.5 % agarose gel, 8 V/cm for 2 hour, MM = Molecular Marker = VII
Lane 1 = POS CONTROL, NEG CONTROL not indicated

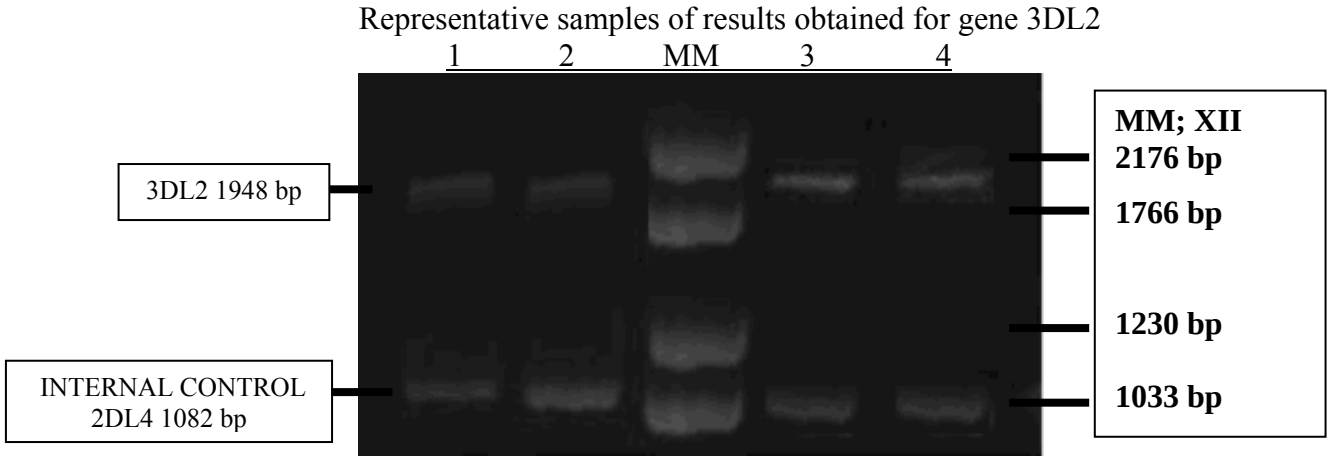

Figure 4.1 (q) Photograph of 3DL2 gene PCR product
1.5 % agarose gel, 8 V/cm for 2 hour, MM = Molecular Marker = XII
Lane 1 = POS CONTROL, NEG CONTROL not indicated

4.3 Gene and haplotype frequencies

In Table 4.5, frequencies for all three cohorts are tabulated under functional headings of inhibitory KIR, activatory KIR and pseudo KIR. Results for the Caucasian cohort[a] in this study are compared to results from Caucasian cohorts[b] accumulated from previous studies (Uhrberg *et al.* 1997, Crum *et al.* 2000, Witt *et al.* 1999, Norman *et al.* 2001, Toneva *et al.* 2001, Niokou *et al*) totalling 698 individuals. Table 4.5 shows the KLFs as calculated per Equation 3.3.

Figure 4.2 depicts haplotype frequency in all three cohorts for the four variable haplotypes. These being homozygous A, homozygous B, heterozygous AB and C for all newly described haplotypes.

Tables 4.9 to 4.11 represent both linkage disequilibrium and Chi^2 data where the most significant results of tables 4.6 to 4.8 were taken. The level of significance (p) is indicated with a star (*) and were taken from Yates contingency table, read across 1 degree of freedom.

Linkage disequilibrium (Δ) values greater than 0.1, were interpreted to indicate positive linkage disequilibrium. While LD values smaller than -0.1, indicated negative linkage disequilibrium. The degree of association is indicated by the relative (Δ') linkage disequilibrium as calculated using Equation 3.6.

Table 4.5 Observed gene percentages and KLF for all three cohorts

		Inhibatory KIR									Activatory KIR						Psuedo KIR	
		2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	2DP1	3DP1
Caucasian [a]	%	94	30	92	100	18	14	96	100	100	52	30	10	92	26	72	98	100
(n=50)	KLF	0.76	0.16	0.72	1.00	0.09	0.07	0.60	1.00	1.00	0.31	0.16	0.05	0.72	0.14	0.47	0.90	1.00
Caucasian [b]	%	91	49	92	100	nt	nt	97	100	nt	45	51	24	96	32	42	nt	100
(n=698)	KLF	0.70	0.28	0.72	1.00	nt	nt	0.83	1.00	nt	0.26	0.30	0.13	0.79	0.18	0.24	nt	1.00

		Inhibitory KIR									Activatory KIR						Psuedo KIR	
		2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	2DP1	3DP1
Mixed Ancestry	%	28	34	48	100	28	34	94	100	100	8	12	24	96	40	50	88	100
(n=50)	KLF	0.15	0.18	0.28	1.00	0.15	0.18	0.76	1.00	1.00	0.04	0.06	0.13	0.80	0.23	0.29	0.65	1.00

		Inhibitory KIR									Activatory KIR						Psuedo KIR	
		2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	2DP1	3DP1
African black	%	82	34	74	100	24	26	94	100	100	30	34	10	98	44	32	98	100
(n=50)	KLF	0.58	0.19	0.49	1.00	0.12	0.13	0.76	1.00	1.00	0.16	0.19	0.05	0.86	0.25	0.18	0.86	1.00

[a] Present Study.

[b] Cumulative data for studies Uhrberg *et al* 1997 [18], Witt *et al* 1999 [17], Crum *et al* 2000 [24], Norman *et al* 2001 [23], Toneva *et al* 2001 [25], Niokou *et al* 2003 [28].

Abreviations: KLF = KIR locus frequency; KIR = killer-cell immunoglobulin-like receptor; n = number; nt = not tabulated.

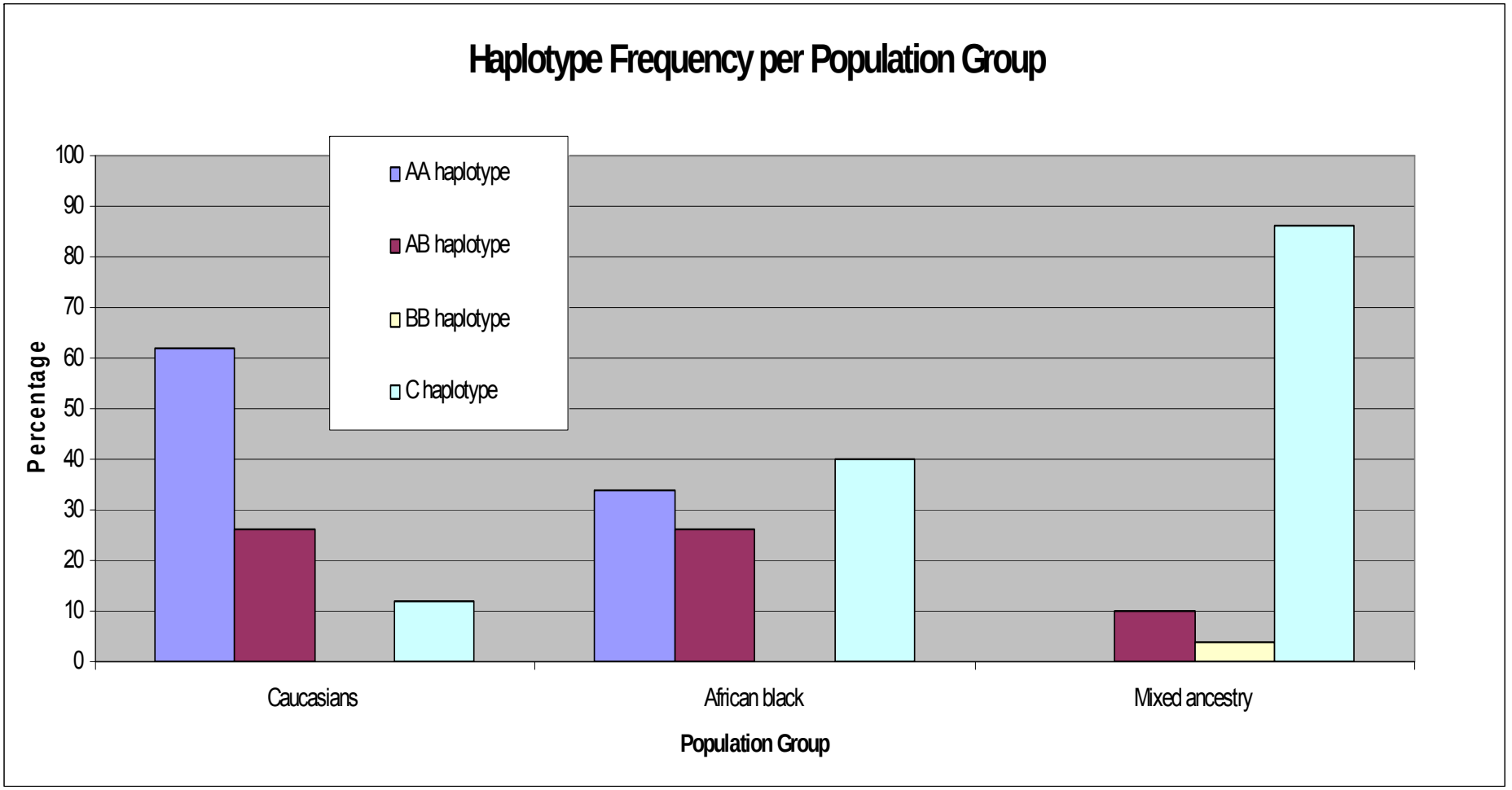

Figure 4.2 Haplotype frequency per population group

Table 4.6 Calculated two-locus haplotype frequency (h), significance (*p*). Relative linkage disequilibrium (r) (threshold = 1), and linkage disequilibrium (Δ) for the Caucasian population

	2DL1	2DL2	2DL3	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DS1
2DL1 h p Δ r		0.001 0.027 0.018 0.06	<0.001 0.013 0.015 0.017	<0.001 0.02 0.01 0.22	<0.001 0.018 - 0.16	<0.001 0.012 0.01 0.39	0.001 0.027 0.018 0.428	<0.001 0.015 0.006 0.111	<0.001 0.013 0.015 0.017	<0.001 0.025 0.015 0.35	<<0.001 0.001 -0.001 0.06	0.021 0.116 0.017 0.019	<<0.001 0.003 0.003 0.19
2DL2 h p Δ r			<0.001 0.024 0.016 0.66	0.08 0.1 0.066 0.066	0.007 0.05 0.018 0.42	0.012 0.05 0.04 0.28	0.49 0.1 0.21 0.428	0.083 0.1 0.05 0.08	<0.001 0.024 -0.016 0.66	0.086 0.1 0.082 0.158	<0.001 0.008 0.00 0.42	0.003 0.044 -0.008 0.666	0.002 0.041 0.024 0.122
2DL3 h p Δ r				0.003 0.05 0.02 0.03	<0.001 0.024 0.01 0.16	0.003 0.044 -0.03 0.92	<0.001 0.024 -0.016 0.66	0.011 0.05 -0.032 0.04	<<0.001 0.005 -0.006 0.15	0.001 0.033 0.02 0.35	<<0.001 0.001 -0.001 0.08	0.014 0.097 -0.003 0.086	<<0.001 0.002 -0.002 0.041
2DL5A h p Δ r					0.025 0.1 -0.025 0.03	0.023 0.1 0.046 0.49	0.08 0.1 0.066 0.115	0.213 0.1 0.062 0.084	<0.001 0.01 -0.005 0.39	0.114 0.1 0.073 0.12	<<0.001 0.006 0.00 0.22	<0.001 0.018 -0.012 0.016	0.019 0.1 0.05 0.388
2DL5B h p Δ r						0.007 0.05 -0.022 0.34	0.007 0.05 0.018 0.42	0.014 0.05 -0.014 0.16	<0.001 0.024 0.011 0.16	0.294 0.1 0.103 0.16	<<0.001 0.006 0.002 0.162	0.004 0.052 0.005 0.162	0.015 0.05 0.039 0.388
2DS1 h p Δ r							0.012 0.05 0.044 0.282	0.015 0.05 0.028 0.53	<0.001 0.021 0.018 0.44	0.014 0.05 0.04 0.33	<0.001 0.011 0.01 0.02	0.009 0.076 0.000 0.041	0.011 0.05 0.065 0.488
2DS2 h p Δ r								0.083 0.1 0.05 0.079	<0.001 0.024 -0.016 0.66	0.086 0.1 0.08 0.15	<0.001 0.008 0.006 0.428	0.003 0.044 -0.008 0.666	0.002 0.041 0.024 0.122
2DS3 h p Δ r									0.001 0.031 -0.012 0.014	0.007 0.05 0.01 0.53	<<0.001 0.005 0.002 0.111	<0.001 0.011 -0.016 0.018	0.01 0.05 0.028 0.388
2DS4 h p Δ r										<<0.001 0.001 0.00 0.01	<0.001 0.001 -0.001 0.08	0.028 0.132 0.036 0.041	<0.001 0.017 0.017 0.244
2DS5 h p Δ r											<0.001 0.008 0.00 0.01	0.002 0.036 -0.009 0.92	0.028 0.1 0.072 0.388
2DP1 h p Δ r												0.016 0.102 -0.000 0.041	<<0.001 0.005 -0.005 0.388
3DL1 h p Δ r													0.014 0.097 0.008 0.785
3DS1 h p Δ r													

Table 4.7 Calculated two-locus haplotype frequency (h), significance (*p*). Relative linkage disequilibrium (r) (threshold = 1), and linkage disequilibrium (Δ) for the Black African population

	2DL1	2DL2	2DL3	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DS1
2DL1													
h		0.006	0.003	<<0.001	0.005	<0.001	0.006	0.003	<<0.001	0.004	<<0.001	0.003	0.001
p		0.062	0.047	0.005	0.057	0.022	0.062	0.05	0.003	0.054	0.003	0.044	0.034
Δ		0.041	-0.046	0.003	-0.033	0.014	0.041	0.018	-0.003	-0.04	-0.003	0.049	-0.022
r		0.673	1	0.074	0.249	0.259	0.673	1	1	0.404	1	1	0.183
2DL2													
h			0.003	0.004	0.001	<<0.001	0.435	0.019	<0.001	0.006	<0.001	0.001	0.024
p			0.045	0.051	0.031	0.005	0.490	0.112	0.009	0.062	0.009	0.028	0.123
Δ			0.028	0.018	0.011	-0.002	0.224	0.026	0.006	0.03	0.006	0.02	0.051
r			0.165	0.116	0.067	0.019	1	0.393	1	0.383	1	1	0.221
2DL3													
h				0.018	0.003	0.007	0.003	0.015	<<0.001	<<0.001	<<0.001	<0.001	0.007
p				0.108	0.05	0.071	0.045	0.099	0.004	0.007	0.004	0.014	0.07
Δ				-0.057	0.027	-0.042	0.028	-0.034	-0.005	-0.005	-0.005	-0.015	0.043
r				0.324	0.408	0.23	0.321	0.459	1	0.038	1	1	0.259
2DL5A													
h					0.062	0.01	0.004	<0.001	<0.001	0.052	<<0.001	0.002	0.017
p					0.197	0.083	0.051	0.02	0.025	0.181	0.007	0.042	0.105
Δ					-0.062	0.028	0.018	-0.004	-0.015	0.0.74	0.004	-0.025	-0.036
r					1	0.166	0.116	0.166	1	0.553	1	0.561	0.239
2DL5B													
h						<<0.001	0.001	0.044	<0.001	0.185	<0.001	<<0.001	0.016
p						0.005	0.031	0.167	0.008	0.333	0.008	0.007	0.101
Δ						0.002	0.011	0.034	0.005	0.145	0.005	-0.004	0.036
r						0.01	0.067	0.447	1	1	1	0.09	0.208
2DS1													
h							<<0.001	0.003	<0.001	<0.001	<0.001	<<0.001	0.002
p							0.005	0.046	0.02	0.017	0.008	0.003	0.041
Δ							-0.002	-0.01	-0.014	0.008	0.006	-0.002	-0.016
r							0.019	0.333	1	0.047	1	0.047	0.083
2DS2													
h								0.019	<0.001	0.006	<0.001	<<0.001	0.024
p								0.112	0.018	0.062	0.018	<0.001	0.123
Δ								0.026	-0.0132	0.03	-0.013	0.000	0.051
r								0.393	1	0.159	1	0.019	0.221
2DS3													
h									<<0.001	0.029	<<0.001	<0.001	0.072
p									0.005	0.136	0.005	0.015	0.211
Δ									0.002	0.036	0.002	0.006	0.048
r									1	0.642	1	1	0.705
2DS4													
h										<0.001	<<0.001	<<0.001	<0.001
p										0.01	<0.001	0.001	0.009
Δ										0.008	-0.000	-0.001	0.006
r										1	1	1	0.5
2DS5													
h											<0.001	0.002	<<0.001
p											0.01	0.041	0.001
Δ											0.008	-0.033	-0.000
r											1	1	0.002
2DP1													
h												<<0.001	<0.001
p												0.001	0.009
Δ												-0.001	0.006
r												1	0.5
3DL1													
h													<<0.001
p													0.001
Δ													-0.000
r													0.019
3DS1													
h													
p													
Δ													
r													

Table 4.8 Calculated two-locus haplotype frequency (h), significance (*p*). Relative linkage disequilibrium (r) (threshold = 1), and linkage disequilibrium (Δ) for the Mixed ancestry population

	2DL1	2DL2	2DL3	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DS1
2DL1													
h		0.16	0.008	0.02	0.013	<0.001	0.02	0.011	<0.001	<0.001	0.004	0.001	0.002
p		0.1	0.05	0.1	0.05	0.012	0.1	0.05	0.017	0.019	0.05	0.026	0.025
Δ		0.124	<0.001	0.041	-0.035	0.002	0.026	-0.027	0.011	0.008	0.016	0.016	-0.02
r		0.675	-0.034	0.206	0.369	0.107	0.333	0.404	1	0.047	0.5	1	0.142
2DL2													
h			0.001	0.02	0.02	0.001	<<0.001	0.005	<0.001	0.004	0.001	0.001	<0.001
p			0.033	0.1	0.05	0.034	0.003	0.05	0.018	0.05	0.003	0.028	0.019
Δ			0.016	0.044	-0.015	-0.007	-0.000	0.021	-0.013	0.024	0.02	0.02	0.01
r			0.095	0.242	0.134	0.264	0.019	0.264	1	0.117	0.5	1	0.058
2DL3													
h				0.02	0.001	0.012	0.005	0.002	<0.001	<0.001	0.001	<0.001	0.001
p				0.1	0.033	0.05	0.05	0.035	0.024	0.014	0.027	0.01	0.032
Δ				-0.054	0.016	0.021	-0.017	-0.015	-0.02	0.008	0.008	0.008	0.02
r				0.404	0.095	0.519	0.305	0.131	1	0.038	0.152	0.305	0.083
2DL5A													
h					0.006	0.02	<0.001	0.016	<0.001	0.02	<0.001	<<0.001	0.011
p					0.05	0.1	0.027	0.05	0.017	0.141	0.021	0.004	0.05
Δ					0.024	-0.022	0.006	0.032	0.011	0.048	-0.003	-0.003	-0.04
r					0.134	1	0.074	0.189	1	0.258	0.037	0.074	0.285
2DL5B													
h						0.001	0.04	0.004	<0.001	0.3	<<0.001	0.011	<0.001
p						0.034	0.1	0.05	0.008	0.1	0.001	0.05	0.019
Δ						-0.007	-0.04	0.018	-0.006	0.204	-0.059	-0.059	0.01
r						0.264	1	0.116	0.242	1	0.752	0.186	0.058
2DS1													
h							0.009	<<0.001	<0.001	0.004	0.001	<0.001	0.01
p							0.078	0.004	0.009	0.05	0.031	0.013	0.079
Δ							-0.009	0.000	0.003	-0.012	0.004	0.004	0.02
r							1	0.013	1	0.375	0.5	1	0.5
2DS2													
h								0.028	<0.001	0.048	0.001	0.001	0.006
p								0.1	0.011	0.1	0.035	0.03	0.05
Δ								0	0.004	-0.048	-0.012	-0.012	0.02
r								-0.028	1	1	0.121	0.242	0.06
2DS3													
h									<0.001	0.02	<0.001	<0.001	0.003
p									0.015	0.1	0.019	0.009	0.046
Δ									0.009	0.044	-0.005	-0.005	0.02
r									1	0.305	0.061	0.122	0.12
2DS4													
h										<0.001	<<0.001	<<0.001	<0.001
p										0.005	0.004	0.002	0.023
Δ										-0.004	-0.002	-0.002	-0.02
r										0.166	0.5	1	1
2DS5													
h											<0.001	0.001	0.002
p											0.01	0.031	0.035
Δ											0.024	0.024	0.02
r											0.5	1	0.333
2DP1													
h												<<0.001	0.00
p												0.006	0.00
Δ												-0.007	0.00
r												1	0.00
3DL1													
h													0.001
p													0.034
Δ													0.03
r													1
3DS1													
h													
p													
Δ													
r													

Table 4.9 (a) Significant linkage-disequilibrium (Δ) for KIR genes within the Caucasian cohort

Positive linkage disequilbrium	Significance
2DL2-2DS2; 2DL5B-2DS5;	*
Negative linkage disequilbrium	
NONE	

Δ > 0.1 = Positive linkage disequilibrium

Δ < -0.1 = Negative linkage disequilibrium

Table 4.9 (b) Significant two-locus association (*p*) for KIR within the Caucasian cohort

Gene Pairs	Significanc
2DL1-2DL2; 2DL1-2DL3; 2DL1-2DL5A; 2DL1-2DL5B; 2DL1-2DS1; 2DL1-2DS3; 2DL1-2DS4; 2DL1-2DS5; 2DL2-2DL3;	*
2DL2-2DS4; 2DL2-3DL1; 2DL3-3DS1; 2DL3-2DL5B; 2DL3-2DS1; 2DL3-2DS2; 2DL3-2DS5; 2DL5A-2DS4; 2DL5A-3DL1;	*
2DS1-2DS4; 2DS1-2DP1; 2DS2-2DS4; 2DS2-3DL1; 2DS2-3DS1; 2DS3-2DS4; 2DS3-3DL1; 2DS4-3DS1; 2DS5-3DL1;	*
2DL2-2DS2; 2DL5B-2DS4:	*
2DL1-2DP1; 2DL1-3DS1; 2DL2-2DP1; 2DL3-2DS4; 2DL3-2DP1; 2DL3-3DS1; 2DL5A-2DP1; 2DL5B-2DP1; 2DS2-2DP1;	**
2DS3-2DP1; 2DS4-2DS5; 2DS4-2DP1; 2DS5-2DP1; 2DP1-3DS1:	**

df = 1 = $p < 0.05$ = *

$p < 0.01$ = **

Table 4.10 (a) Significant linkage-disequilibrium (Δ) for KIR genes within the Black African cohort

Positive linkage disequilbrium	Significance
2DL2-2DS2; 2DL5B-2DS5;	*
Negative linkage disequilbrium	
NONE	

Δ > 0.1 = Positive linkage disequilibrium

Δ < -0.1 = Negative linkage disequilibrium

Table 4.10 (b) Significant two-locus association (*p*) for KIR within the Black African cohort

Gene Pairs	Significanc
2DL1-2DL3; 2DL1-2DS1; 2DL1-3DL1; 2DL1-3DS1; 2DL2-2DL3; 2DL2-2DL5B; 2DL2-3DL1; 2DL3-2DS2; 2DL3-3DL1;	*
2DL5A-2DS3; 2DL5A-2DS4; 2DL5A-3DL1; 2DL5B-2DS2; 2DS1-2DS3; 2DS1-2DS4; 2DS1-2DS5; 2DS1-3DS1;	*
2DS2-2DS4; 2DS2-2DP1; 2DS3-3DL1; 2DS4-2DS5; 2DS5-2DP1; 2DS5-3DL1:	*
2DL1-2DL5A; 2DL1-2DS4; 2DL1-2DP1; 2DL2-2DS4; 2DL2-2DP1; 2DL5A-2DP1; 2DL5B-2DS1; 2DL5B-2DS4;	**
2DL5B-2DP1; 2DL5B-3DL1; 2DS1-2DS2; 2DS1-2DP1; 2DS1-3DL1; 2DS2-3DL1; 2DS3-2DS4; 2DS3-2DP1; 2DS4-2DP1;	**
2DS4-3DL1; 2DS4-3DS1; 2DS5-3DS1; 2DP1-3DL1; 2DP1-3DS1; 3DL1-3DS1; 2DL3-2DS4; 2DL3-2DS5; 2DL3-2DP1:	**

df = 1 = $p < 0.05$ = *

$p < 0.01$ = **

Table 4.11 (a) Significant linkage-disequilibrium (Δ) for KIR genes within the Mixed ancestry cohort

Positive linkage disequilbrium	Significanc
2DL1-2DL2; 2DL5B-2DS5;	*
Negative linkage disequilbrium	
NONE	

Δ > 0.1 = Positive linkage disequilibrium

Δ < -0.1 = Negative linkage disequilibrium

Table 4.11 (b) Significant two-locus association (*p*) for KIR within the Mixed ancestry cohort

Gene Pairs	Significance
2DL1-2DL3; 2DL1-2DL5B; 2DL1-2DS3; 2DL1-2DP1; 2DL1-3DS1; 2DL2-2DL5B; 2DL2-2DS3; 2DL2-2DS5;	*
2DL3-2DS2; 2DL5A-2DL5B; 2DL5A-2DS3; 2DL5A-3DS1; 2DL5B-2DS3; 2DL5B-3DL1; 2DS1-2DS5;	*
2DL3-2DS1; 2DS2-3DS1:	*
2DL1-2DL2; 2DL1-2DL5A; 2DL1-2DS2; 2DL2-2DL5A; 2DL3-2DL5A; 2DL3-2DS5; 2DL5A-2DS1;	**
2DL5A-2DS5; 2DL5B-2DS2; 2DL5B-2DS5; 2DS2-2DS3; 2DS2-2DS5; 2DS3-2DS5;	**

df = 1 = $p < 0.05$ = *

$p < 0.01$ = **

CHAPTER 5

Discussion and Conclusion

5.1 Introduction

In this study three South Africa ethnic cohorts were genotyped for KIR genes. Of the 150 samples, 64 new haplotypes have been observed and are nominated as C-haplptypes in Table 4.4, 42 which were found in the mixed ancestry population, as well as 22 new haplotypes found in the Black African population and six in the Caucasian cohort. Four of these haplotypes occurred in multiple cohorts as represented by CC45 in Table 4.4.

The first KIR genes to be standardised and genotyped using the in-house method were the framework genes (2DL4, 3DL2, 3DL3, 3DP1). This allowed for the initial process refinement and functioned as a pilot study. Tabulated results in chapter 4 show the presence of a 100 % for the three framework genes as well as the pseudo-gene 3DP1. Initial findings concurred with previously reported results and can be seen in Table 4.5 (Uhrberg *et al.* 1997, Crum *et al.* 2000, Witt *et al.* 1999, Norman *et al.* 2001, Toneva *et al.* 2001, Niokou *et al.* 2003).

Framework genes (KIR2DL4 - 1084 bp) showed normal PCR products throughout the genotyping process with the exception of five individuals of the mixed ancestry (PCR product not pictured), where abnormal, smaller, lighter intensity PCR products fragments (<1084 bp) were observed for the 2DL4 gene. Suspicious product, fragments were smaller (<1084 bp) than the normal detected 2DL4 (1084 bp) fragments and are speculated to be a mutated gene missing a fragment ultimately resulting in a smaller PCR fragment (<1084 bp) for the duplicated gene. Upon further investigation small fragments was not generated in repeat experiments. Therefore these samples were placed aside with there results discarded and marked for inclusion in follow up studies which will make use of more specific genotyping methods and sequencing.

After genotyping of the framework genes, the genes at the other loci were typed. It was decided that the framework genes would be suitable as internal controls due to the availability, as well as the positive presence of these genes in all samples. For the long fragments ranging from 1000 kb to 2000 kb, 2DL4 was used as an internal control, and for the shorter fragments smaller than 1000 kb, 3DL3 or 3DP1 were used depending on the yield of duplex reactions. Standardisation for all KIR genes were done first as a simplex reaction and then as a duplex reaction where gene specific fragments and internal control fragments were required to amplify their respective genes. A balance was reached between the product yield for gene specific and internal control in order to provide equal PCR opportunity.

5.2 General problem solving

Quality of DNA samples also played a significant role throughout the study. During the initial standardisation and genotyping, samples were exposed to repetitive freezing, thawing and vortexing before use in PCR. This resulted in DNA fragmentation and was noticed when long PCR fragments failed to amplify. DNA extraction of initial blood samples also proved problematic due to old age, clumping in the blood and the routine discarding of blood samples from previous years. Due to these problems the initial cohort samples and results were discarded. New blood samples were collected from paternity clinic cases, fresh DNA isolated from the new samples, followed by the identification of new population profiles.

DNA quality of the initial batch was not the only PCR difficulty encountered in the initial cohort study; certain primer pairs failed to yield any PCR product in the standardisation process. Poor design, suitability towards specific populations or gene specificity may have contributed to these primers not annealing. After completion of standardisation, new primers were ordered for those which failed in the initial reactions and were PCR successfully there after. These primers were for KIR2DS5, KIR3DL2 and KIR2DL3 (Hiby *et al.* 2004, Gómez-Lozano and Vilches *et al,* 2002).

5.3 KIR locus frequencies

Table 4.5 indicates frequencies for all KIR genes in the three ethnic populations. Also included in Table 4.5 are the estimated gene frequencies for the putative KIR loci (KLF) as calculated via Equation 3.3. Results from South African Caucasians are similar to those reported in previous studies investigating 90 individuals from Ireland (Crum *et al.* 2000), 136 individuals from the UK (Norman *et al.* 2001), 52 individuals from the USA (Uhrberg *et al.* 1997), 147 individuals from Australia (Witt *et al.* 1999), 40 individuals from Australia (Toneva *et al.* 2001) and 233 individuals from Greece (Niokou *et al.* 2003) while noticeable difference occur at some loci (2DL2, 2DS2, 2DS3 and 3DS1). Further comparisons can also be drawn between South African Cuacasians, African blacks and mixed ancestry, where similarities as well as noticeable differences can be observed.

Overall the inhibitory receptors occur more frequently than their activatory counterparts and results are consistent with previous findings as reported by Niokou *et al.* (2003). A Balance between inhibitory and activatory genes is expected as inhibitory receptors are indispensable and a wide range of inhibition activities towards antigens are required to prevent unnecessary cell lysis. One can observe in Table 4.5 that the most prevalent inhibitory receptor is 3DL1 for Caucasians (96 %) while for mixed ancestry and Black African populations 3DL1 is present in 94 % of individuals tested.

Inhibitory receptors with the lowest possessed percentages include 2DL5B (14 %) in Caucasians and 28 % for 2DL5A and 2DL1 in mixed ancestry, while 2DL5A were possessed at 24 % in the Black African cohort. Further observations revealed that some individuals lack all inhibitory receptors specific for HLA-C Class I ligands (2DL1/2DL2/2DL3) and is a novel finding in the present study. Uhrberg *et al.* (1997), Gumperz *et al.* (1996) and Niokou *et al.* (2003) mention individuals who lacked the inhibitory receptor 3DL1 specific for the HLA-Bw4 antigen. In the present study, 8 individuals (5 %) (AB8, CC24, CC25, CC42, CC44, CC61, CC63 and CC64) did not possesses 3DL1 which is consistent with their findings. Furthermore, two individuals (CC24, CC25) in the mixed ancestry cohort lacked the inhibitory receptor KIR3DL1, as well as 2DL1, 2DL2 and 2DL3 receptors which have never been reported in previous studies.

Most activating receptors are found at lower percentages as is evident by the observations of 2DS3 (10 %) in African blacks and Caucasians, while 2DS1 (8 %) is the least frequent activating receptor for the mixed ancestry cohort. These findings for the Caucasian population are consistent with results from Uhrberg *et al.* (2002) who reported a 2DS3 frequency of 20 %, making this their least possessed receptor.

Receptor 2DS4 is the most frequently possessed activating receptor for all three cohorts and is present in Caucasian, Black African and mixed ancestry at percentages of 92 %, 96 % and 98 % respectively. Comparative investigation of activating receptors between cohorts revealed a 44 % difference between South African Caucasians and mixed ancestry for their 2DS1 receptors, highlighting some of the vast differences between these two cohorts.

While some individuals have been noted to lack most major inhibitory receptors, the same is true for activating receptors where one individual (haplotype CC61) from the Caucasian cohort has been identified to lack all activating receptors. Reports from Hsu *et al.* (2002b) also state that certain A homozygous individuals do not possess activating receptors, while this is the case for the mentioned individual (CC61), this sample is classified as a C-haplotype, distinguishing this profile from pervious reports.

Results comparing 3DL1 and 3DS1 revealed that 52 % of all individuals harboured both these genes and only 3 % of the individuals lacked both of these genes. This finding is unexpected as both these genes are alleles of the same locus, occupying the same position. Looking at linkage disequilibrium results for these two genes no great variation was observed, indicating normal inheritance patterns within South African cohorts. Although previous reports have been made of individuals possessing both these genes have been reported previously, the percentage described here is greater than previously reported (Caucasian, $\Delta = 0.008$; black African, $\Delta = 0.000$; mixed ancestry $\Delta = 0.03$) (Crum *et al.* 2000, Gardiner *et al.* 2001).

5.4 Complex reasoning

Haplotype profiles in Table 4.4 show a vast amount of variation with relative low percentage for all cohorts. The most frequent profile observed is haplotype AA3 that

was detected in 15 individuals (10 %), 7 from the Black African cohort and 8 from the Caucasian cohort. This haplotype contains 6 inhibitory receptors with only the one activating receptor 2DS4, encompassing a total of 9 genes. Niokou *et al.* (2003) also reported this haplotype (AA3) as their most prevalent with 24.4 % of their reported individuals possessed this haplotype, which draws comparison between these two studies. In contrast to their study, high frequency with limited variability was not detected in our cohort. The next most common haplotype detected is AA6 with a percentage of 5.3 %.

Niokou *et al.* (2003) and Norman *et al.* (2001) both reported AA3 and AB1 in high frequencies, while AA3 was the most prevalent in the present study, the combined prevalence of these two haplotypes was 12 %. Other haplotypes were present in noticeable lower percentage throughout the three cohorts

Haplotype AA6 was the second most frequent haplotype with a frequency of 6 % and was present in 6 Caucasians and 2 Black African individuals. Niokou *et al.* (2003), Norman *et al.* (2001) and Witt *et al.* (1999) did not report this haplotype in investigations involving Greek, English and Australian Caucasians thus indicating a unique finding in the present study. On average, 11 genes were present in the observed haplotypes with the maximum number being 15 and the minimum being 6. Haplotype variability as noted in Table 4.4 indicates that there are 93 different haplotypes throughout the three cohorts, demonstrating the polymorphic and polygenic nature of the KIR gene family. Both homozygous and heterozygous A and B-haplotypes can be noted in Table 4.4, as well as haplotypes missing 2DL1, 2DL2, 2DL3 (C-haplotypes). In Black African and Caucasians cohorts 48 of these haplotypes are found while the mixed ancestry cohort harbours the remaining 45 haplotypes.

As seen in Figure 4.2 the AB-haplotype is fairly evenly represented between the Caucasian and Black African populations indicating 26 % of each cohort. Within the Caucasian cohort the homozygous A-haplotype was the most prevalent and occurred with a percentage of 62 %. Several other studies also show that the A-haplotype is more prevalent within Caucasian populations reiterating the similarities between these Caucasian populations on a global level (Norman *et al.* 2001, Witt *et al.* 1999, Crum *et al.* 2000, Toneva *et al.* 2001). No Caucasian individuals that were homozygous for the B-haplotype, were indicated. Other novel haplotypes with in the Caucasian

population include a frequency of 12 % for the C-haplotypes these include C2, C11, C21, C28, C45 and C61 haplotypes.

Homozygous B individuals were also not found in the Black African population, while homozygous A individuals and heterozygous AB individuals are closely comparable with 34 % and 26 % respectively. Unlike Caucasians the Black African cohort possessed 40 % C-haplotypes, all of which are novel KIR haplotypes.

By far the most surprising haplotype percentages are seen in the mixed ancestry population, where 86 % of individuals displayed the C-haplotype. Additionally, the mixed ancestry population did not harbour any homozygous A-haplotypes while only 2 homozygous B-haplotype individuals were found. Heterozygous AB-haplotypes were the other great contributor towards variability within the mixed ancestry cohort showing a percentage of 10 %.

Estimated linkage disequilibrium calculations (Equation 3.4) were done to determine the inheritance equilibrium of two-locus KIR pairs for each of the three cohorts. As noted in Tables 4.6 to 4.8, the linkage disequilibrium (Δ) indicates the positive and negative two-locus inheritance between gene pairs.

Results for the Caucasian population indicate positive associations between 2DL2-2DS2 and 2DL5B-2DS5. Our data confirm the report by Norman *et al.* (2001) stating that 2DL2 was found in linkage disequilibrium with 2DS2. Similar findings between 2DL5B-2DS5 was not mentioned in previous studies but are present in all three South African cohorts indicating no linkage disequilibrium between these two genes for all three cohorts. One can thus affirm, that within all individuals 2DL2-2DS2 and 2DL5B-2DS5 are inherited together or absent together.

For the Black African population only two pairs of genes in LD were seen, these include 2DL2-2DS2, and 2DL5B-2DS5. Both these pairs show positive linkage disequilibrium thus indicating that these genes always appear on the same haplotypes. While only one of the above mention pairs (2DL2-2DS2) were observed for the Caucasian cohort thus indicating novel findings.

Furthermore, the mixed ancestry cohort, showing positive linkage disequilibrium between 2DL1-2DL2 and 2DL5B-2DS5 genes and are novel to this study. Interesting findings are the constant positive association between 2DL5B and 2DS5 throughout the three cohorts, as well as positive association between 2DL2-2DS2 for black African's and Caucasians. No negative linkage was reported for any of the three cohorts indicating no negative bias towards two genes being inherited together.

Results for Chi^2 from all three cohorts showed several significant differences between observed and expected frequencies. These were not anticipated when comparing results from previous studies. The most significant two-locus association was observed for 2DS4 and 3DL1, across Tables 4.6 to 4.8. These patterns of significance were observed in the Caucasian cohorts and are unique findings within these populations. Plausible reasons for the genetic drift observed within the populations may include the emerging of viral pathogens. It would be expected that the KIR locus would compensate and evolve in relation with HLA. One can also argue increased contact between South African populations with individuals from other countries allowing more opportunities to acquire gene material.

Findings for the Caucasian and Black African populations showed relative association between observed and expected results indicating more stable gene pools within these cohorts. While one might assume that the present AIDS epidemic would drive the KIR repertoire into a mode of compensation and evolution, lack of survival from this disease does not allow the opportunity for appropriate stabilisation of the KIR repertoire, thus a greater degree of drift is possible.

The objective of the study was to develop and standardise an in-house KIR genotyping method and to compare results to those obtained from Prof Derrek Middleton. A database for South African cohorts would also be constructed. Standardised processes were conclusively developed for the detection of the 17 recognised KIR genes thus fulfilling that part of the project aims. Furthermore, gene frequencies for the cohorts were determined and a significant number of new haplotypes detected when compared to previous studies (Uhrberg *et al.* 1997, Crum *et al.* 2000, Witt *et al.* 1999, Norman *et al.* 2001, Toneva *et al.* 2001, Niokou *et al.* 2003).

For the Caucasian cohort similarities between the present study and previously reported studies indicate some discrepancy as well as some similarities. Much of the variation was observed within the mixed ancestry and Black African populations where these populations would require a large follow up study with increased sensitivity and specificity for confirmation of reported results.

5.5 Critical reasoning

Unexpected haplotype variability has been noticed within this population when compared to other studied cohorts. The author assumes that a possible explanation for this vast variability observed in the mixed ancestry population is the fact it is such a heterogeneous population being descendants from Khoi-Khoi, Malay, San, European, Black African.

Other factors which need to be included and considered are population size, where only a limited number were included in the present study. It is envisaged that with larger population sizes, similar frequencies of the same haplotypes would be expected. Further investigations are also recommended to confirm negative results as well as false positive results via more specific methods such as sequence specific oligo-nucleotide primer-polymerase chain reaction as there is no such thing as a perfect study.

The role KIR plays in disease can be pursued by investigating ways in which activatory KIR/HLA interactions can be manipulated in order to obtain favourable outcomes. Such examples includes possible vaccines, allowing for blocking the HLA process and ultimately leading to susceptibility of target cells

Furthermore, NK cells and KIR can also be manipulated and suppressed as to avoid autoimmune disease states such as rheumatoid and psoriatic arthritis (Martin *et al.* 2002a). Ways in which this can be accomplished include regulating interleukin levels which in turn regulates cell cytotoxicity.

5.6 Conclusion

In conclusion, we have demonstrated the procedures for in-house genotyping of KIR, as well as the construction of a database for three population groups within central South Africa. Through our investigations new haplotypes have been observed, demonstrating the polymorphic nature of KIR. Further results also indicate that the three population groups are similar and that no major discrepancies can be noted. With this project we have added to current understanding and comprehension of the immune system, as well as understanding the central role NK cells play in immunity. Overall, understanding of KIR goes a long way in understanding the innate immune response.

CHAPTER 6

Opsomming

In die meegaande projek is daar ’n ondersoek gedoen na die interval voorkoms van KIR gene in drie Suid Afrikaanse studiegroepe. KIR speel ’n belangrike rol in immuniteit deur die vulling van is gaping tussen virus besmetting en sel-gemediëerde immuniteit. As gevolg van die vermoë om abnormale selle waar te neem, besit moordselle die vermoë om selle te vernietg wanneer ’n abnormale HLA molekule uitgedruk word tydens KIR/HLA interaksie.

Swart, kleurling en blanke etniese groepe is ingesluit in die studie. DNA is van indiwidue verkry deur gebruik te maak van die “uitsout” proses voor SSP-PCR genotipering. Sewentien inleiër pare was gebruik in die identifisering van die individuele KIR gene. Die elektroforese van die PCR produkte is dan opgeweeg teen ’n molekulêre gewigsaanwyser om die korrekte grootte te bepaal. Die eindprodukte is besigtig onder ’n ultraviolet lig en aangeteken as teenwoordig al dan nie.

Die inligting is aangeteken in ’n tabel met die aanduiding van elke geen as teenwoordig al dan nie. Hierdie tabelle is dan gebruik vir grafiese en Chi^2 verwerking. Hierdie grafieke is gebruik vir ’n kritiese analiese van koppelversteurings binne die etniese groepe.

Die resultate bevestig dat al die raamwerkgene 100 % teenwoordig is binne al drie groepe. Die swart en kleurling groepe is nie voorheen vir KIR tipeer nie. Ondersoek binne die nie-raamwerk gene het gelei tot die ontdekking van nuwe haplotipes, waarvan die meeste waargeneem is binne die kleurling groep. Positiewe koppelversteurings is gevind tussen 2DL2-2DS2 en 2DL5B-2DS5 vir beide die blanke en swart groepe terwyl 2DL1-2DL2 en 2DL5B-2DS5 koppelvesteurings binne die kleurling groep vertoon het. Geen negatiewe koppel versteurings is waargeneem binne enige van die groepe nie.

CHAPTER 7

Summary

In the foregoing project, an investigation was made into the relative KIR gene frequencies of three South African cohorts. Playing an important part in innate immunity, KIR fill a vital gap between viral onsets and cell mediated humeral immunity. Being able to sense when cells are abnormal, NK cells possess the ability to destroy cells which show altered HLA molecules during KIR/HLA interaction.

Ethnic cohorts that were investigated included African black, mixed ancestry and the Caucasian populations. From these individuals DNA material was extracted using a "salting out" method before SSP-PCR genotyping. Seventeen primer pairs were used in the identification of individual KIR genes. PCR products were electrophoresed against a molecular weight marker in order to verify the correct fragment size. Products were viewed on a UV light where observations were noted, and indicated as present or absent.

Data was recorded onto a spreadsheet indicating the absence or presence of each particular gene. Tabulated results were used in the construction of graphs as well as χ^2 calculations. These graphs were used in the critical analysis of linkage disequilibrium as well as comparative analysis between the ethnic cohorts.

Findings indicate that all framework genes are present in all cohorts. The Black African and mixed ancestry cohorts have not been genotyped for the KIR genes before. Investigation within non-framework genes revealed the identification of several new haplotypes, with the majority observed within the mixed ancestry cohort. Positive linkage disequilibrium was detected between 2DL2-2DS2 and 2DL5B-2DS5 for both the black African and Caucasian cohorts while 2DL1-2DL2 and 2DL5B-2DS5 linkages were found in the mixed ancestry population. No negative linkages were observed for any of the three cohorts.

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